Pergamon

Biochemical Pharmacology, Vol. 47, No. 1, pp. 3–13, 1994.

0006-2952/94 $6.00 + 0.00

FROM RECEPTOR INTERNALIZATION TO NUCLEAR TRANSLOCATION

NEW TARGETS FOR LONG-TERM PHARMACOLOGY

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Abstract—Receptors involved in intercellular communication at the cell surface share the capacity to desensitize through molecular and cellular mechanisms. Cellular desensitization is a rapid and dynamic process whereby membrane receptors internalize in response to an excess of agonists. The internalized receptors may recycle rapidly or undergo down-regulation when following a degradative pathway. However, receptor internalization does not necessarily mean degradation; it also represents the initial step of a retrograde signalling system whereby an "interiorized" message, the ligand–receptor complex, can be transported in contrast to second messengers, along axons or in the cytoplasm leading to long-term effects in the nucleus. Such "third messengers" have to undergo nuclear translocation to serve as transcriptional regulators in the control of gene expression. The "third messengers" are thus cytoplasmic proteins, including the receptor itself, which may be associated with internalized vesicles and released by mechanisms which have not yet been elucidated. They represent already good targets for the development of new drugs, and multitargeting and synergistic approaches are likely to increase their usefulness.

Key words: receptor internalization, desensitization, axonal transport, microtubule, third messenger, nuclear translocation, gene expression

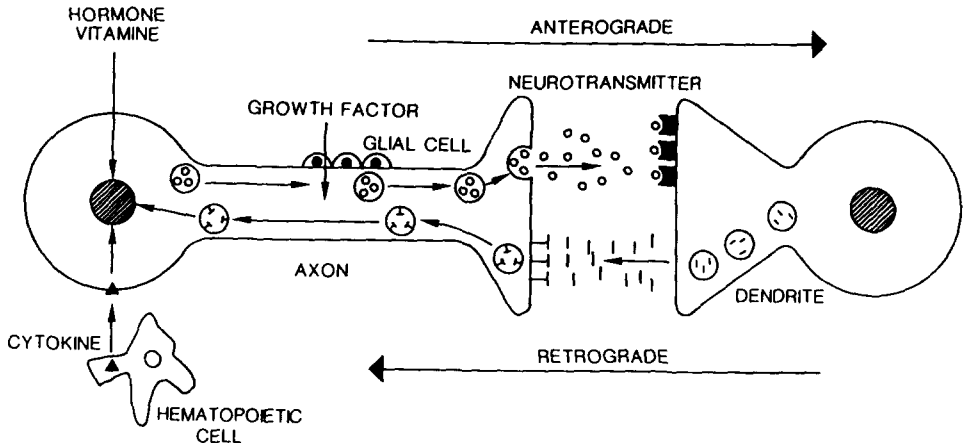

Fig. 1. Schema illustrating intercellular communication between different types of cell through anterograde and retrograde signalling systems.

The most striking property of living cells is communication between themselves via a great variety of signals and receptors. Cells as different as those of hematopoietic, glial and neuronal origin (Fig. 1) are capable of exchanging information via cytokines, growth factors, neuropeptides and classical neurotransmitters. Practically all the cells possess receptors at the cell surface and in the nucleus. Hormones (steroid, thyroxine) and vitamins (retinoic acid, vitamin D3) belong to a special class of signalling systems because they can readily penetrate the cell membranes to interact directly with nuclear receptors [1, 2].

Membrane receptors are more complex because they have to convey a signal from the exterior to the interior of the cell since the chemical transmitter or ligand does not cross the cell membrane. This inability to penetrate the cell has a great advantage

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as it provides for greater selectivity and close connection in the dialogue between cells. Indeed, when transmitters are released into the synaptic cleft, only postsynaptic receptors are activated so that the intercellular communication is, here, a kind of "personal conversation" between cells in contrast to the scattered effects of corticoid hormones which affect numerous cells.

Pharmacologists have learned to make a clear distinction between the short-term and long-term effects of drugs. As a rule, compounds acting on nuclear receptors bring about long-term responses by changes in gene expression. This has been called "genomic" pharmacology [3]. However, drugs acting on membrane receptors, can also effect gene expression through a cascade of intracellular events, such as the production of second messengers, but the role of these has probably been overestimated.

Different stimuli can also induce long-term effects in cells especially under pathological conditions. An example of this is the peripheral noxious stimulus which activates nociceptors, the primary sensory neurones, and leads to release of substance P in the spinal cord [4]. The consequence of this is an immediate increase in c-Fos mRNA followed later by a marked rise in dynorphin that persists for at least one day [5]. Of course c-Fos is not a specific marker of pain but the specificity of the response resides in the group of neurones that are affected by the noxious stimulus, in the spinal cord as well as at the supraspinal level in the thalamus and cortex.

The aim of the present paper is to discuss some recent data which support the idea that membrane receptors can, like nuclear receptors, lead to changes in gene expression through a complex mechanism involving agonist-induced internalization of membrane receptors, cytoplasmic or axonal transport of internalized vesicles, and nuclear translocation of "third messengers", which can play the role of transcriptional regulators at gene level.

Receptor desensitization

The notion that receptors are involved in desensitization is as old as the receptor concept. Indeed it has been known for a long time that when membrane receptors are exposed to a relatively high concentration of agonists, they become refractory to their action. This may have consequences in therapy, as for instance in asthmatic patients treated with β-adrenergic agonists, β-adrenergic receptors desensitize making the patients refractory to further drug treatment. Desensitization is called homologous or ligand-specific when the receptor is only desensitized by its ligand. By contrast, when the cell becomes refractory to a larger range of ligands as well as to non-receptor-mediated stimuli (e.g. phorbol esters), the desensitization is termed heterologous but this will not be discussed here.

Several mechanisms have been invoked to explain the desensitization of receptors, but a clear distinction has to be made between what is called molecular and cellular desensitization. Molecular desensitization is due to agonist-induced conformational changes as is the case for the nicotinic receptor, a pentameric protein. In its desensitized form, the nicotinic receptor is a closed channel and displays a higher affinity (10 nM) for nicotinic ligands [6]. This type of desensitization seems to be common to all the multimeric ligand-gated ion channels. Cellular desensitization involves mechanisms such as receptor internalization, uncoupling, phosphorylation and down-regulation. These of course also have a molecular basis. Sometimes, the term desensitization has been misused as when used to describe the inhibitory effects of a high concentration of neuropeptides like substance P and CGRP* [7, 8]. In fact, this pseudodesensitization was due to the formation of metabolites in particular with a high concentration of neuropeptides. This may explain why some neuropeptides display bell-shape biological responses which can be normalized by using peptidase inhibitors.

The term desensitization was also used to describe the decrease in adenylate cyclase observed when β-adrenergic receptors were exposed to agonists in intact cells [9, 10]. In fact, the cyclase activity was measured in membrane fractions and not intact cells. This point is crucial for interpretation of the data. In stimulating cyclase in membrane fractions, isoproterenol may affect receptors associated with cell membranes as well as those contained in vesicles. Indeed, isoproterenol was found to stimulate the formation of cAMP in the sciatic nerves of the frog and rat, and in the vagus nerves of the rat [11, 12], where β-adrenergic receptors are in vesicles since they undergo rapid axonal transport like many other receptor sites [13–15]. Moreover, the β-adrenergic receptors transported anterogradely along axons are of high affinity and thus GTP sensitive, a fact which indicates functional coupling between receptor and G-protein [14, 16].

Therefore, in the experiments on cyclase desensitization, the problem of receptor internalization was not taken into account. To overcome this difficulty, one should treat intact cells with hydrophilic agonists, to make sure that they only interact with cell surface receptors, and then estimate the cAMP formation in these cells. Before this can be achieved, the measurement of cyclase in plasma membranes derived from agonist-treated cells cannot be taken as an index of receptor desensitization. Moreover, the β-adrenergic receptor of the turkey erythrocyte which appears rather as a remnant is not physiologically relevant to the study of the regulation of neuronal receptors. In any case, the decrease in cyclase activity may be explained in several ways as the different environmental conditions of the receptors in membranes and internalized vesicles lead to conformational changes. A similar explanation may account for the loss of accessibility to isoproterenol in vesicles and the progressive acidification of internalized vesicles going from pH 6–6.5 for "early" endosomes to pH 4–4.56 for late endosomes [17]. This latter point may be important since second messengers were found to regulate endosomal acidification [18].

* Abbreviations: CGRP, calcitonin gene-related peptide; IL, interleukin; EGF, epidermal growth factor; NGF, nerve growth factor; TNF, tumor necrosis factor; NLS, nuclear localization sequence.

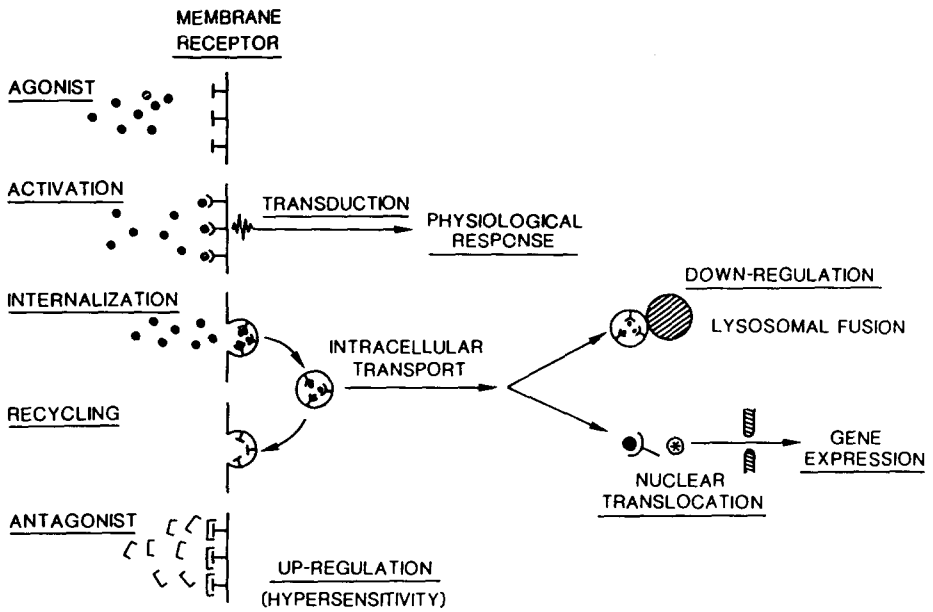

Fig. 2. Model for the regulation of membrane receptors. Agonist-induced receptor internalization may lead to recycling or down-regulation of receptors, or to nuclear translocation of third messengers for regulation in gene expression. Antagonists cause hypersensitivity or up-regulation of receptors.

Receptor internalization in cellular desensitization

G-protein-coupled receptors or serpentine receptors (with seven transmembrane domains in the form of a serpent) as well as receptors not coupled to G-proteins desensitize through processes involving agonist-induced receptor internalization (Fig. 2). Sometimes the term sequestration has been used as in the field of β-adrenergic receptors for which it is believed that receptor internalization is not a major mechanism underlying rapid desensitization [9]. Nevertheless, the internalization process has been demonstrated for a wide variety of receptors of neuropeptides, cytokines, growth factors and classical neurotransmitters.

Receptor internalization which corresponds to the disappearance of receptors from the cell surface is rapid (few minutes), induced by agonists, blocked by antagonists, and temperature and agonist concentration dependent [19]. Antagonists increase the number of receptors at the cell surface (Fig. 2) in a process termed receptor hypersensitivity. Transferrin, low-density lipoprotein and asialoglycoprotein receptors are internalized in a ligand-independent manner. This is not surprising: these receptors have no transduction systems that allow the transmission of signals from the exterior to the interior of the cell. Similarly, the uptake of peroxidase in nerve terminals is non-specific but requires the integrity of nerve electrical activity in contrast to the ligand-induced internalization process which occurs independently of the electrical activity.

Since ligand-induced receptor internalization is an agonist-dependent process it is easy to understand why pharmacological approaches are not only essential for the study of receptor internalization but represent a great advantage over the use of morphological or biological techniques [20]. In this regard, the development of hydrophilic ligands like [^{3}H]CGP 12177 and [^{3}H]methylscopolamine for measurement at the cell surface of β-adrenergic and muscarinic receptors, respectively, has been used to demonstrate genuine and rapid disappearance of receptors from cell membranes [21, 22]. Before these ligands became available, receptor desensitization was thought to be due to a decrease in the total number of receptors measured *in vitro* by means of binding assays. In fact, this represents what is now called receptor down-regulation. This is a late event appearing after prolonged (several hours) exposure of membrane receptors to agonists and is the consequence of receptor internalization and of a degradation process resulting from the fusion of internalized vesicles with lysosomes (Fig. 2) (cf. Ref. 19). Therefore, the model of cellular desensitization presented in Fig. 2 involves agonist-induced receptor internalization, recycling or down-regulation of the receptor site.

The internalization process concerns not only the receptor or binding site but also the G-protein and the effector. The classical notion that the G-protein and the effector as adenylate cyclase are not associated with the receptor site during the internalization into vesicles [9, 23, 24] is probably not correct. Indeed while the trimeric G-proteins have been suggested to be cytoplasmic shuttles [25], they need detergents to be solubilized [26]. Moreover, they are transported axonally in both antero- and retrograde directions [27, 28] and are associated with synaptic [14, 16] and pinocytotic vesicles [29].

The fact that carbachol activation was found to induce a parallel down-regulation of muscarinic

receptors and the α subunit of G-protein, a process which was clearly due to an enhancement of degradation rather than to an increase in synthesis [30, 31], strongly supports the idea of a close association within the membranes of receptor sites and G-proteins. Therefore, the dissociation of both receptor sites and G-proteins during agonist-induced internalization is unlikely. However, this does not exclude a possible uncoupling which is a mechanism whereby G-proteins release a GTP-α subunit from the receptor after binding of agonists in order to activate the effector [32]. Normally, this lasts a few seconds; just enough to amplify the signal. That a more persistent uncoupling occurs during the internalization is quite plausible. However, since the receptors not coupled to G-proteins also internalize through a ligand-induced process, this persistent uncoupling seems to be the consequence of receptor internalization rather than the cause. This could be due to phosphorylation of receptors [33]. There is also evidence that phosphorylation is involved in directing internalized receptors towards the degradation pathway. The substitution of tyrosine residues (phosphorylation sites) in the carboxyl tail of the human β_2-adrenergic receptor did not affect receptor internalization but prevented subsequent down-regulation [34]. Similarly, a mutant of EGF and macrophage colony stimulating factor receptors with an inactive kinase underwent ligand-induced internalization but failed to be down-regulated [35, 36]. From these data, one may conclude that agonist-induced receptor internalization represents the major mechanism for the cellular desensitization of receptors.

Receptor internalization triggering

Receptors are not continuously influenced by submaximal concentrations of ligand as under stress or pathological conditions. For receptors coupled to G-proteins, desensitization appears as a consequence of the high efficacy of the amplification systems. In cell cultures, high concentrations of agonists and prolonged exposures both increase the formation of second messengers and enhance the internalization process. However, second messengers are not necessary to initiate agonist-induced receptor internalization; first, because this process also occurs in receptors not coupled to G-protein and second because a β-adrenergic receptor mutant with a small sequence of the M_1 muscarinic receptor (amino acid 220–230) failed to activate either adenylate cyclase or phospholipase C but was able to mediate receptor internalization [37]. If therefore second messengers are not needed, the third loop of the β-adrenergic receptor that binds to the G-protein is definitely required. The deletion of residues 222–229 from the hamster β-adrenergic receptor resulted in inability of the mutant receptor to undergo the agonist-mediated internalization response [37]. Similarly, deletion of only one single domain 284–292 of the third loop of the muscarinic receptor impaired agonist internalization [38]. In contrast, the carboxy terminal tail of the hamster β-adrenergic receptor is not required for receptor internalization [39] but this is not the rule for all the serpentine receptors. Altogether, these results suggest that coupling to G-protein plays a major role in agonist-induced receptor internalization. Why? Presumably because of the high affinity state that receptors are in when they are coupled to G-proteins. The low affinity site represents binding of agonist to receptors when G-proteins are bound to GTP or GDP. The high affinity state, which is transient, results from conformational changes that accompany receptor activation by agonists. The activated receptor has thus high affinity for the agonist and for the G-protein in which the single guanine nucleotide binding site of the α-subunit is empty. Interestingly, the binding of agonists to β-adrenergic receptors is temperature dependent [40] as is the agonist-induced receptor internalization, while the binding of antagonists is relatively independent of temperature.

In the anterograde direction, the receptors transported axonally are of high affinity, but they are of low affinity in the retrograde direction [14, 16]. Therefore, it is not surprising that the number of high affinity sites in neuronal cells is quite variable from 20 to 80%, a number which is rather similar to the number of receptors undergoing internalization. In most cells, the number of available G-proteins appears to limit the number of receptors that can form a high affinity complex with agonists, even if a single occupied receptor can probably interact with multiple G-proteins [41]. The human M_2 and M_3 muscarinic, α_2-adrenergic and $5HT_{1A}$ receptors when transfected into intact cells mediate both inhibition of adenylate cyclase and stimulation of phospholipase C [41].

It is not surprising that the number of high affinity sites is limited; in guinea pig ileum for example, occupancy of less than 0.25% of the muscarinic receptors by acetylcholine is sufficient to cause a half-maximal contraction [42], whereas in rat brain, less than 1% occupancy of opiate receptors elicits analgesia.

If the high affinity state of serpentine receptors seems to be required for initiating agonist-induced receptor internalization, this does not mean that the heterotrimeric G-proteins serve as triggering signals in this process. Indeed, receptors not coupled to G-proteins may also internalize. To solve this dilemma we put forward the working hypothesis that small G-proteins can be involved in mechanisms underlying receptor internalization. There is no direct experimental basis, but there exists a number of experiments which show that small G-proteins are operational in numerous cellular functions including ribosomal protein synthesis, membrane traffic, polymerization of tubulin exocytosis and even endocytosis [43–46].

Small G-proteins or GTP binding proteins belonging to the ras super family are monomeric in contrast to the large hetero-trimeric G-proteins. Rab_1 and rab_2 have been shown to control vesicular transport from the endoplasmic reticulum to the Golgi complex [43, 45]. Formation of secretory vesicles through budding from the trans-Golgi network was inhibited by non-hydrolysable analogues of GTP [46]. Rab_3 is associated with chromaffin granules [47] and synaptic vesicles [48], and seems to be implicated in the transmitter secretion involved in controlling exocytosis in mast cells and pituitary

cells [49, 50]. A GTP binding protein should direct the docking of synaptic vesicles to the presynaptic membrane [51]. Interestingly, trimeric G-proteins were also found to be associated with secretory granules as well as with plasma membranes, suggesting a possible role in the regulation of exocytosis [52]. Taken together, these results demonstrate that G-proteins play a major role in the secretory pathway from vesicle formation and vectorial transport of secretory granules to fusion with plasma membranes. Conversely, small G-proteins are also involved in the endocytotic pathway. Early endosome fusion that follows receptor-mediated internalization was blocked by GTP-γ-S, a non-hydrolysable GTP analogue indicating that GTP hydrolysis has to take place to trigger this process [53].

Rab_4 and rab_5 which are associated with early endosomes and also localized in the cytoplasmic face of the plasma membrane seem to be the small G-proteins that control the early endosome fusion events [54, 55]. The fact that rab_4 was also found to be involved in the intracellular transport of transferrin receptors suggests that this small G-protein plays a role in regulating the pathway of receptor recycling [54]. Finally, another GTP binding protein is dynamin, which was first identified as a microtubule binding protein [56]. Microtubules, anionic phospholipids and some SH domains are thought to bind to dynamin within the carboxy-terminal basic region.

Interestingly, the gene encoding dynamin is mutated in shibire, a *Drosophila* mutant defective in synaptic vesicle recycling in neurons and more generally in endocytosis from the plasma membrane. When overexpressed in transfected cultured cells, mutant forms of mammalian dynamin were also shown to block receptor-mediated endocytosis (see Ref. 57). More recently it was reported that phosphorylation of dynamin by protein kinase C enhances 12-fold its GTPase activity [58]. It is tempting to see in dynamin a link between the endocytotic pathway and the receptor signal transducing systems. Taken together these data suggest a possible involvement of small G-proteins in mechanisms leading to agonist-induced receptor internalization. Since both trimeric and small G-proteins are involved in budding and vesicular transport in the Golgi network, one may assume that a similar process occurs at the level of plasma membranes after activation of numerous receptors.

Fate of internalized receptors

After internalization, several receptors recycle rapidly to reappear at the cell surface [19]. There is no clear explanation why receptors endowed with a micromolar affinity recycle more readily than those with nanomolar affinity. The receptor affinity is not the only criterion since transferrin receptors which are of high affinity recycle continuously, but in this case the internalization process can occur in the absence of ligands.

The assumption, sometimes ranked as a dogma, that endocytosis equals degradation constitutes, with the second messengers, the major argument levelled against the idea that receptor internalization can play a key role in intracellular signalling. Of course, internalized receptors may undergo down-regulation through degradative pathways but they can serve also in the process of long-distance signalling to elicit the long-term effects of ligands like cytokines, growth factors and neuropeptides [3, 59]. In this regard, it is essential to make a distinction between the short-term and long-term effects of ligands. In neurons, short-term effects are confined to nerve terminals and performed by mechanisms operating in the vicinity of membrane receptors (Fig. 3). For example, opiate inhibits substance P release in nerve endings of sensory neurones while neurotensin increases dopamine release through presynaptic receptors located on dopaminergic nerve terminals. However, both may also display long-term effects: opiate decreases substance P synthesis [60] and neurotensin increases mRNAs of tyrosine hydroxylase in the cell body of dopaminergic neurons after retrograde axonal transport [61, 62]. Of course, this requires a prior internalization process at the nerve terminals. Such ligand-mediated internalization was clearly demonstrated with neurotensin (see Ref. 63). However, the idea that internalization of ligand-bound receptors is necessary for long-term effects is just emerging. Indeed, second messengers were believed to be the signal for eliciting biological responses of NGF because of the misleading interpretation of experiments with NGF and NGF antibodies which, when injected into cells, were ineffective in inducing or preventing the effects of NGF produced outside the cells [64]. Of course, the antibodies could not reach NGF internalized within vesicles. Recent experiments clearly showed that the overexpression in PC_{12} cells of gp140trk, the NGF high affinity receptors, accelerated NGF-induced differentiation [65] and that altered internalization of NGF resulted in signalling dysfunction [66].

There are, today, many other systems in which ligand-mediated receptor internalization is required to elicit long-term responses such as the antitumor effect of TNF [67] or the ability of IL-1 to activate the genes of the IL-2 receptor [68] and of IL-8 [69]. Sometimes receptor internalization may occur under the form of a multimeric complex as with the α and β chains of the high affinity IL-2 receptor [70] or the homodimers as is the case for EGF [71]. As a rule the cytoplasmic region of receptors and even a restricted domain of the C terminal region seem to play a key role in the internalization process as well as in further processing, leading to gene expression as found for IL-1, IL-2, IL-4, EGF, platelet-derived growth factor and erythropoietin receptors [72–77].

Short-term and long-term effects appear to be mediated via different systems (Fig. 3). In this regard it is noteworthy that short-term effects of EGF like calcium increase are independent of receptor internalization as shown with mutants, which were unable to increase the intracellular calcium concentration, but were well able to undergo receptor internalization [73]. Two ligands as different as NGF and EGF may share common short-term biological effects but exert totally different long-term responses such as cell division for EGF and neurite outgrowth for NGF [78].

Obviously, ligand-mediated receptor internalization that escapes the degradative pathways can

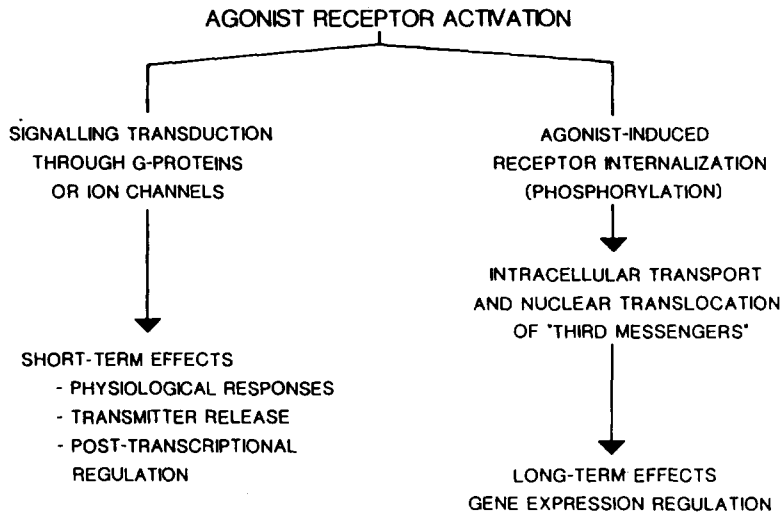

Fig. 3. Schema illustrating the two levels whereby agonist receptor activation may induce short- and long-term effects.

yield cellular responses with a greater specificity than can second messengers. In a sense, the message, here, becomes "interiorized" through a process which appears to be the most economical for the cell and highly selective because it is receptor dependent. Consequently, endocytosis may lead to positive outcome in addition to its role in the degradative pathways. However, the latter will always remain the most apparent.

Intracellular transport

The neuron is an ideal model for studying *in vivo* the intracellular transport of receptors because of the extraordinary eccentricity of nerve terminals with regard to the cell body (Fig. 1). Indeed the distance between the perikaryon and presynaptic receptors may be a few microns to several millimetres or centimetres, going even to one meter as in human sciatic nerves. In contrast, outer membranes of non-neuronal cells are separated from the nucleus by as little as 20 nm.

In the absence of protein synthesis in axons and nerve terminals, all the proteins including neuropeptides must be exported from the cell body to the periphery. Axonal transport was first reported for muscarinic receptors [13] and then for many other sites in the periphery as well as in the brain [14–16, 59, 62, 79]. Anterograde transport makes presynaptic receptors operational at nerve terminals while in the retrograde direction, receptors convey signal molecules to the cell body [15, 59].

NGF was first reported to undergo retrograde axonal transport when NGF receptors had not yet been identified [80]. Opiate receptors labelled *in vivo* with [^{3}H]-lofentanil were also found to move retrogradely in sensory neurones [81, 82]. However, this synthetic compound may also bind opiate receptors in the neuron cell body. Therefore, the finding that labelled neurotensin was transported retrogradely from the striatum to the substantia nigra provides more direct evidence for the existence of a retrograde signalling system [61, 62, 79]. More recently, fibroblast growth factor (FGF), leukemia inhibitory factor (LIF), BDNF and NT-3 were also reported to move retrogradely in axons in periphery or in the brain [83–86]. Interestingly, the differentiative response to NGF and bFGF and the neurite proliferation with NGF was prevented by orthovanadate, an inhibitor of protein tyrosine phosphatase which also can affect axonal transport [87]. Let us recall that second messengers may not undergo retrograde axonal transport so that free cytoplasmic molecules do not move back to the cell body. Therefore, the role of second messengers following activation of presynaptic receptors remains only confined to the nerve terminals thus for the short-term effects (Fig. 3). Nitric oxide (NO) was also called a retrograde signal [88] but its action is not only quite transient but confined to the nerve terminals in the vicinity of plasma membranes. NO should be termed transynaptic signal rather than retrograde signal.

The integrity of cytoskeleton is a prerequisite for axonal transport. Microtubule inhibitors, as for example colchicine and vinblastine may impair the retrograde transport of signal molecules [62, 82]. In cell cultures, colchicine or cytochalasin B prevent receptor down-regulation without affecting the internalization process [19]. This is compatible with the notion that early endosome fusion is not facilitated by microtubules *in vitro* in contrast to the later endocytotic fusion event [89]. Interestingly, taxol, a promising anti-cancer drug was found to counteract colchicine blockade of axonal transport presumably owing to its property to hyperpolymerize tubulin in microtubule while colchicine has the opposite effect [90]. Colchicine, a very ancient drug, is commonly used in the treatment of gout. Recently, very low doses of colchicine were reported to inhibit plasma extravasation induced by electrical stimulation [91]. This suggests that inhibition of axonal transport of substance P may contribute to the anti-inflammatory and analgesic effect of the drug. But the most impressive action of colchicine

was reported in the familial mediterranean fever in young children. The dose of 1 mg/day given chronically resulted in complete remission or marked reduction in the frequency of attacks and even an increase in survival [92]. Colchicine is also effective in liver cirrhosis and pericarditis [93].

In immunohistochemistry, colchicine has been commonly used to identify neuropeptides in the cell bodies and seems to produce the same effects as axotomy as for example up-regulation of galanin and vasoactive intestinal peptide and down-regulation of substance P and CGRP [94]. More surprising is the finding that colchicine had neuroprotective properties in the gerbil model of cerebral ischemia [95]. If colchicine protects neurons against deleterious signals, it is not surprising that chronic administration of the drug resulted in a dose-dependent learning deficit [96].

Intracellular transport may be of prime importance in controlling cell function, but as yet there are only a few examples of the beneficial effects of colchicine, presumably because of its toxicity, which is not solely related to its antimicrotubular action. The cytoskeleton still remains a good target for drug action; examples are the benzimidazole carbamates belonging to the class of antihelmentic drugs and the antineoplastic drugs, vincristine and vinblastine, and the new class, taxol and taxotere. Microtubule dynamics have become much better understood: tubulin, which is composed of α and β subunits, is also a GTP protein [97]. The nature of microtubule-associated proteins is different in dendrites and axons belonging to the microtubule-associated protein and tau peptide family, respectively [97].

The neuropeptides that serve as trophic substances [94] are more abundant than those belonging to the growth factor family. These peptides as the cytoskeleton itself may play a role in the pathogenesis of neurodegenerative diseases.

Nuclear translocation of third messengers

The question is now as to how signals originating from receptor internalization and intracellular transport may affect gene expression in the nucleus. Of course, this is a crucial step which presumably involves nuclear translocation of "third messengers" able to serve as transcriptional regulators in the nucleus. The nuclear translocation process of proteins through nuclear pores begins to be better understood [98, 99], but the major problem resides in the mechanisms allowing proteins firmly associated with internalized vesicles to leave the vesicular membrane to undergo nuclear translocation. At present, there is no clear answer to this problem. As already discussed [3] the occurrence of short basic NLSs in the intracytoplasmic tail of some membrane receptors is compatible with the idea that the receptor itself or a part of it may undergo nuclear translocation. Supporting this view is the recent experiment with an artificial protein consisting of the SV40 large T antigen containing a NLS, coupled to human serum albumin and rhodamine [100]. When injected into the axoplasm of Aplysia neurons *in vitro*, the complex was transported retrogradely along the axon to the cell body and then into the nucleus. Interestingly, the NLS, here, provides access to both the retrograde transport and the nuclear translocation. As discussed previously [3] and further in this symposium, numerous neuropeptides, cytokines and growth factors were recovered in the nucleus sometimes even with their receptors.

Three proteins associated with the interferon α receptor and termed ISGF proteins were found to undergo nuclear translocation after phosphorylation which occurs when the receptor is activated with interferon α [101]. In the nucleus, these three proteins (113, 91 and 84 kDa) serve as transcriptional activators through binding to interferon-stimulated response elements in DNA [101]. In addition to the classical transcription factors Jun, Fos and NF-kB, NF-IL-6 was recently identified as a transcriptional regulator involved in the induction of IL-6 by IL-1 and in the expression of acute phase protein [102]. The promoters of the IL-1, IL-6, IL-8 and TNF genes contain binding sites for NF-IL-6 [102].

A collection of papers was published recently and at the same time on the cascade of events following EGF receptor activation. Although numerous proteins (GRB_2, SOS, RAS, RAF-1, MEK, MAPK) seem to be involved depending on the specific receptor and the cell type, the link between cytoplasmic events and nuclear transcriptional activation has not yet been clearly elucidated except for the SIF protein [103].

"Third messengers" can, therefore, be considered to be all the proteins including the receptor itself which are recruited for phosphorylation through receptor activation and serve as transcriptional regulators after nuclear translocation. When receptors possess only a short intracellular tail, they are not good candidates for third messengers. This could explain why receptors with a short intracellular segment need to be associated as dimers. The protein gp-130 of the IL-6, leukaemia inhibitory factor and ciliary neurotrophic factor receptors is an example of such a receptor-associated protein [102]. One may speculate that a proteolysis step as recently reported for the inhibitory subunit IkB of the transcriptional activator protein NK-kB [104] can take place at the level of internalized vesicles to release third messengers.

In the last two years, more evidence has emerged that nuclear receptors become more effective as heterodimers in modulating gene expression. Thus, the retinoic acid receptor, in particular in its RXR form, was found to form heterodimers with thyroxine, vitamin D_3 and peroxisome proliferator-activated receptor (PPAR) leading to enhanced gene expression [2], and new heterodimers await discovery. Third messengers in the form of a heterodimer could simply be the consequence of multiple transmitters that coexist in the same neuron, which implies the co-release of transmitters and co-internalization of receptors.

A possible consequence of heterodimerization for therapy is the synergistic effects of compounds. The dramatic antitumor activity of 13-*cis* retinoic acid in combination with recombinant interferon α2a in cancers of the cervix and skin [105, 106], and the efficacy of interferon α with cytotoxic chemotherapy in patients with non-Hodgkin's lymphoma are two examples of such synergism [107]. Similarly, a

synergistic effect of the thrombin inhibitor and the platelet aggregation inhibitor as well as of the thromboxane A synthetase blocker was reported in the models of experimental thrombosis [108, 109].

Future prospects

There is today more evidence that the intracellular signalling systems involve the transfer of signals from cell membranes to the nucleus via agonist-induced receptor internalization, transport of vesicles and nuclear translocation of "third messengers". The major problem remaining resides in the identification of mechanisms necessary to dismantle internalized vesicles to yield "third messengers".

The main objective in genomic pharmacology is to reveal drugs which are able to block the long-term effects of ligands in preventing their short-term effects. In hypertension, for instance, the short-term effects of noradrenaline had to be maintained in order to prevent orthostatic hypotension. Monotherapy is often insufficient to treat patients because of the multifactorial origin of chronic diseases, and also because of the diversity of signalling transduction systems which allow the cell to overcome the blockade of a single process. Multitargeting drugs and drugs acting synergistically are needed in modern therapy for the treatment of asthma, arthritis, arteriosclerosis and of the neurodegenerative diseases, as well as of schizophrenia and depression. If the concept of synergism can be further explored in various pathological areas, one may expect to have drugs which are "inactive" when used alone but become active in synergy with another compound. Synergism is also involved in whole body responses, such as angiogenesis, neurotoxicity, inflammation and nociception.

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