CRITIQUE

PHOSPHORYLATION OF RECEPTORS AND ION CHANNELS AND THEIR INTERACTION WITH STRUCTURAL PROTEINS

Johannes W. Hell
Department of Pharmacology, University of Wisconsin, 3770 MSC, 1300 University Avenue, Madison, WI 53706-1532, U.S.A.

(Received for publication 21 March 1997)

INTRODUCTION

The role of protein tyrosine phosphorylation in the regulation of cell growth and cell cycle as well as cell differentiation has been investigated intensively within the last two decades (Hunter, 1996). Receptor-type protein tyrosine kinases (RTK) possess an extracellular ligand binding domain, a single transmembrane region and a cytosolic catalytic domain. They are usually activated by ligand-induced dimerization and subsequent tyrosine autophosphorylation. Specific phosphotyrosine residues bind SH2 or PTP domains of various proteins, which participate in different signaling pathways thereby recruiting various components of these pathways to the plasma membrane (Schlessinger, 1994; Kavanaugh et al., 1995; Hunter, 1996). Non-receptor-type protein tyrosine kinases (PTK) do not possess a transmembrane region, although several non-receptor PTKs are involved in transmembrane signaling. For example, focal adhesion kinase (Fak) is localized at focal adhesions, where it is associated with the cytoplasmic tail of integrins which interact through their extracellular domains with extracellular matrix proteins such as fibronectin and with their cytosolic regions with the cytoskeleton. Fak is activated upon integrin binding to fibronectin and may subsequently phosphorylate cytoskeletal proteins such as paxillin (Meredith et al., 1996; Otey, 1996).

An increasing body of evidence now suggests that tyrosine phosphorylation may have functions in addition to assembling various signal-transducing components around PTKs localized in or at the plasma membrane and to concomitantly activating signaling cascades. In neurons, for example, PTKs control the activity of different ion channels and neurotransmitter receptors. N-methyl-D-aspartate (NMDA)-type glutamate receptors are associated with the non-receptor PTK Src, which increases the activity of NMDA receptors by tyrosine phosphorylation (Yu et al., 1997). Pyk2, another non-receptor PTK highly expressed in the nervous system, becomes tyrosine-phosphorylated and thereby activated upon Ca\(^{2+}\) influx, muscarinic stimulation or stimulation of protein kinase C (PKC) by phorbol esters (Lev et al., 1995; Siciliano et al., 1996). Pyk2 has been shown to contribute to a suppression of the activity of the delayed-rectifier K\(^+\) channel Kv1.2 when coexpressed in oocytes (Lev et al., 1995). In vitro experiments using tyrosine-phosphorylated, activated Pyk2 immunoisolated from angiotensin II stimulated rat liver epithelial cells (Earp et al., 1995) and immunoisolated NMDA receptors did not reveal phosphorylation of NMDA receptors by Pyk2, nor did these experiments indicate that Pyk2 binds to NMDA receptors (Leonard and Hell, unpublished results). However, stimulation of different G protein-coupled receptors by lysophosphatidic acid or bradykinin in the pheochromocytoma cell line PC12 induces both tyrosine phosphorylation of Pyk2 and association of Pyk2 with Src, which is stimulated by Pyk2 overexpression (Dikic et al., 1996). It is possible that Pyk2 regulates NMDA receptors in intact neurons by activating Src, although this hypothesis has not been tested yet. Regulation of NMDA receptor activity and of other synaptic functions by PTKs are summarized in the preceding review article (Gurd, 1997). In the following sections I will focus on a particular aspect of protein phosphorylation with relevance to synaptic functions, the specific localization of neurotransmitter...
receptors and ion channels at postsynaptic sites. I will
discuss how this localization might be regulated by
phosphorylation and how interaction with structural
proteins might regulate the activity of postsynaptic
ion channels.

THE ACETYLCOLINE RECEPTOR AT THE
NEUROMUSCULAR JUNCTION

The RTK prototype is the epidermal growth factor
(EGF) receptor, which when activated stimulates cell
growth and proliferation through a pathway involving
Ras, Raf, MEK, and MAP kinases (Bonfini et al.,
1996). ErbB2/Neu, ErbB3 and ErbB4, all members of the
EGF receptor family, are concentrated at synaptic
sites (Zhao et al., 1995) together with their ligands,
the neuregulins (Chu et al., 1995; Jo et al., 1995).
Neuregulins are secreted proteins of about 45 kDa,
which may be associated with the extracellular matrix
in the synaptic cleft (Carraway III and Burden, 1995).
They are involved in regulating differentiation of the
neuromuscular junction. They stimulate the
expression of acetylcholine receptors (AChR) and Na+
channels as these postsynaptic channels are tran-
scribed in myofiber nuclei located close to the synapse
(Corfas and Fischbach, 1993; Carraway III and
Burden, 1995; Chu et al., 1995; Jo et al., 1995).

MuSK, another RTK, is activated by agrin, a pro-
teoglycan of the synaptic basal lamina which causes
AChRs to cluster (Tsao et al., 1992; Denzer et al.,
1995; Tsao et al., 1995). MuSK may, therefore,
mediate agrin-induced aggregation and coincident
tyrosine phosphorylation of AChR (Gillespie et al.,
1996; Glass et al., 1996). As mentioned above, phos-
phorylation of individual tyrosine residues of RTKs or
RTK-associated proteins creates docking sites for proteins
involved in the downstream signaling of
RTKs (Bonfini et al., 1996; Hunter, 1996). It is, there-
fore, tempting to speculate that phosphorylated, but
not unphosphorylated, tyrosine residues of AChR
subunits constitute sites for specific interactions with proteins which regulate the subcellular localization
of these receptors, possibly by mediating interactions
between AChR and the cytoskeleton. In support of
such a model are findings that precisely correlate
agrin-induced, staurosporine-sensitive tyrosine phos-
phorylation and clustering of AChR with an increase
in the resistance of AChR to detergent extraction
(Wallace, 1994). Similarly, the tyrosine-phosphatase
inhibitor pervanadate increases tyrosine phos-
phorylation and decreases detergent solubility and
agrin-stimulated clustering with identical time
courses, and also diminishes the lateral mobility of
AChRs, possibly by strengthening the interaction of
diffusely distributed AChRs and the cytoskeleton
(Meier et al., 1995; Wallace, 1995). These findings
support a diffusion-trap model predicting that AChR
are tyrosine-phosphorylated by relevant kinases
which could specifically be localized at postsynaptic
sites. AChRs are then trapped in the vicinity of these
kinases by phosphorylation-dependent interaction
with structural proteins. Such a diffusion-trap model
implies that AChRs are not permanently fixed to
postsynaptic structures but can disperse upon dephos-
phorylation. In fact, removal of agrin from muscle
cell cultures causes de-aggregation of AChRs. This
disintegration of AChR clusters is inhibited by block-
ing tyrosine phosphatases with pervanadate (Wallace,
1995) and promoted by inhibiting protein kinases with
staurosporine (Wallace, 1994), suggesting that consti-
tutive, ongoing phosphorylation is required for the
maintenance of agrin-induced AChR aggregation.

The adapter protein Grb2, which is known to bind
with its SH2 domain to specific phosphorytrosine resi-
dues of various RTKs and with its SH3 domain to
other proteins involved in downstream signaling of
RTKs, has recently been shown to associate with the
tyrosine-phosphorylated but not with non-phos-
phorylated δ subunit of AChRs (Colledge and
Floehner, 1997). Because Grb2 also associates with
β-dystroglycan (Yang et al., 1995), a protein necessary
for the structural integrity of muscle cells and thought
to be involved in AChR clustering (Fallon and Hall,
1994; Hoch et al., 1994), it is quite possible that Grb2
links tyrosine-phosphorylated AChRs to β-dystro-
glycan. β-Dystroglycan possesses a transmembrane
region and is associated with extracellular α-dystro-
glycan. Both proteins are derived from a common
precursor (dystroglycan) by posttranslational proteo-
lytic processing. A 43 kDa protein named rapsyn co-
clusters AChRs and dystroglycan when all three pro-
teins are co-expressed in fibroblast cell lines (Apel
et al., 1995). Rapsyn is, therefore, an alternative can-
date linking AChR with the dystroglycan complex.
Although α-dystroglycan binds agrin (Bowe
et al., 1994; Campanelli et al., 1994; Gee et al., 1994;
Sugiyama et al., 1994), it does not appear to be the
agrin receptor mediating agrin-induced tyrosine phos-
phorylation and clustering of AChRs (Meier et al.,
1996). Dystroglycan may, therefore, not function as a
signaling element but as a structural protein. Accord-
ingly, the interaction between α-dystroglycan and
agrin may contribute to the aggregation of proteins
at neuromuscular junctions by direct protein–protein
interactions rather than by inducing tyrosine phos-
phorylation, which depends on MuSK.
It has been reported that tyrosine phosphorylation of AChR has also a more direct functional effect: it increases the rate of a decline in channel openings over a time period of about 1 min, which was interpreted as desensitization (Hopfield et al., 1988). However, the physiological relevance of this effect is unclear and run-down effects of the isolated AChRs have not been ruled out in this study.

INTERACTIONS BETWEEN POSTSYNAPTIC ION CHANNELS AND THE CYTOSKELETON IN THE BRAIN

As discussed in the previous section for AChRs at neuromuscular junctions, ligand- or voltage-gated ion channels may become clustered at postsynaptic sites through their specific interaction with postsynaptic structural proteins. A single report based on fluorescent photobleach recovery of cultured cortical neurons labeled with a fluorescent ligand for NMDA receptors suggested that NMDA receptors which are not clustered at postsynaptic sites diffuse freely within the plasma membrane (Benke et al., 1993). Similar findings have been reported from this group for the glycine receptor (Srinivasan et al., 1990). Postsynaptic aggregation of the glycine receptor depends on its interaction with the structural protein gephyrin and on an intact cytoskeleton (Kirsch et al., 1993; Kirsch and Betz, 1995; Kuhse et al., 1995). These observations support the idea that neurotransmitter receptors are clustered at postsynaptic sites of central synapses through their interaction with structural proteins.

Within the last two years several structural postsynaptic proteins have been shown to interact with NMDA receptors (reviewed by Garner and Kindler, 1996; Gomperts, 1996; Sheng and Kim, 1996). A yeast two-hybrid screen with NMDA receptor sequences as baits indicated that PSD-95 (Cho et al., 1992) also known as SAP90 (Kistner et al., 1993) interacts with most of the NMDA receptor subunits which are homologous to each other (Kornau et al., 1995). Other members of the PSD-95/SAP90 family, collectively known as synapse-associated proteins (SAPs), include SAP97 (Müller et al., 1995), SAP102 (Müller et al., 1996), and PSD-93/chapsyn-110 (Brenman et al., 1996; Kim et al., 1996). The interaction of NMDA receptor subunits and SAPs is mediated by the three amino acids at the very C-terminal end of each NMDA receptor subunit (S/TXV; S/T: serine or threonine; X: any residue; V: valine). SAPs also interact with other ion channels, especially voltage-gated K+ channels including Kv1.1, 1.2, 1.3, and 1.4 (Kim et al., 1995) and Kir2.1 and 2.3 (Cohen et al., 1996), which possess the same (Kvl.1–4) or a very similar (Kir2.1 and 3; SXI; I: isoleucine) C-terminal motif. Important from a functional point of view is the finding that NMDA receptors and K+ channels, when overexpressed alone in heterologous cell lines, are evenly distributed in the plasma membrane, but form clusters when coexpressed with either PSD-95/SAP90 or PSD-93/chapsyn-110 (Kim et al., 1995, 1996; Sheng and Kim, 1996).

More recently it has been shown that α-actinin, which interacts with actin in a Ca2+ dependent manner, binds to the C0 domain in the C-terminal tail of NMDA receptor subunit NR1 (Wyszynski et al., 1997). In the presence of Ca2+, this interaction was disrupted by calmodulin (Wyszynski et al., 1997), which binds to the C1 domain and, like α-actinin, also to the C0 domain, thereby inhibiting the activity of NMDA receptors (Ehlers et al., 1996). The integrity of actin filaments is crucial for the functional properties of NMDA receptors (Rosenmund and Westbrook, 1993). Ca2+, which depolymerizes actin, causes a fast run-down of the NMDA receptor activity. Actin filaments can be stabilized by the aldehyde phalloidin and by ATP, and both compounds prevented the Ca2+-induced run-down (Rosenmund and Westbrook, 1993). Because calmodulin inhibits NMDA receptors and disrupts the interaction of NMDA receptors with α-actinin in the presence of Ca2+, it is conceivable that α-actinin links NMDA receptors to actin filaments. Accordingly, both Ca2+-dependent direct binding of calmodulin and a disturbance of the actin cytoskeleton may inhibit NMDA receptors.

It is quite possible that the binding of a structural protein to NMDA receptors modulates the activity of the latter directly. Such a mechanism would keep NMDA receptors and other ion channels active mainly at sites where their activity is desired, the postsynaptic sites in the case of neurotransmitter receptors. Another ion channel that is specifically clustered at postsynaptic sites is the class C L-type Ca2+ channel (Hell et al., 1996). Similar to NMDA receptors, the activity of different types of Ca2+ channels (Johnson and Byerly, 1993, 1994) including class C L-type channels as measured in ventricular cells (Galli and DeFelice, 1994) is decreased when microtubules or actin filaments are depolymerized and stabilized when microtubules or actin filament depolymerization is inhibited. However, interaction with structural proteins could also promote phosphorylation of postsynaptic NMDA receptors or class C L-type Ca2+ channels which then causes an increase in activity. As discussed above, the NMDA receptor is associated
with Src and phosphorylation by Src increases its activity (Yu et al., 1997). Tyrosine phosphatases reduce NMDA receptor currents (Wang and Salter, 1994). PTKs also upregulate L-type Ca\(^{2+}\) channels in pituitary GH3 cells and this effect is reversed by tyrosine phosphatases (Cataldi et al., 1996). PTKs are, therefore, potential candidates for the upregulation of ion channel activity upon interaction with structural proteins.

NMDA receptor subunits are also phosphorylated by second messenger-activated serine/threonine kinases including Ca\(^{2+}\) and calmodulin-dependent protein kinase II (CaMKII) (Omkumar et al., 1996), cAMP-dependent protein kinase (PKA) (Leonard and Hell, 1997; Tingley et al., 1997) and PKC (Tingley et al., 1993; Hall and Soderling, 1997; Leonard and Hell, 1997). NMDA receptor responses are potentiated upon PKC activation in trigeminal (Chen and Huang, 1991; Chen and Huang, 1992), spinal cord dorsal horn (Gerber et al., 1989), and hippocampal neurons (Aniksztejn et al., 1992; Wang et al., 1994a,b; but see Markram and Segal, 1992), as well as in oocytes injected with total mRNA (Kelso et al., 1992; Urushihara et al., 1992) or with RNA encoding different subunits of the NMDA receptor (Kutsuwada et al., 1992; Durand et al., 1993; Mori et al., 1993). PKA enhances NMDA receptor activity in spinal dorsal horn neurons (Cerne et al., 1993) and in neuronal microcultures from the hippocampus (Raman et al., 1996). Furthermore, phosphatase 1 and 2A are involved in downregulation of NMDA receptor activity (Lieberman and Mody, 1994; Wang et al., 1994b; Tong et al., 1995). Similarly, the class L-type Ca\(^{2+}\) channel is a substrate for PKA, PKC, CaMKII and cGMP-dependent kinase (PKG) in vitro (Holl et al., 1993, 1994) and for PKA and PKC in intact hippocampal neurons (Holl et al., 1995; Hell, unpublished results). The activity of L-type Ca\(^{2+}\) channels is increased upon PKA stimulation in GH3 cells (Armstrong and Eckert, 1987), guinea-pig hippocampal neurons (Gray and Johnston, 1987; Fisher and Johnston, 1990), rat nodose ganglion neurons (Gross et al., 1990), chromaffin cells (Artalejo et al., 1990), and rat neostriatal neurons (Surmeier et al., 1995). More specifically, the current through class C L-type channels is significantly potentiated by PKA injection when expressed in CHO cells (Sculptoreanu et al., 1993). PKC increases neuronal L-type Ca\(^{2+}\) channel currents in frog sympathetic ganglion neurons (Yang and Tsien, 1993).

Taken together the findings summarized in the preceding paragraphs demonstrate that NMDA receptors and class C L-type Ca\(^{2+}\) channels can be phosphorylated by PTKs as well as by the major arginine/lysine-directed serine/threonine kinases. Several of these phosphorylation sites are tonically phosphorylated as predicted by a model that assumes that the activity of postsynaptic ion channels is constitutively upregulated upon binding to cytoskeletal proteins. Tyrosine residues are phosphorylated tonically on NMDA receptors (Moon et al., 1994; Wang and Salter, 1994) and on L-type Ca\(^{2+}\) channels (Cataldi et al., 1996). Serine/threonine residues corresponding to PKA, PKC or CaMKII phosphorylation sites are constitutively phosphorylated on NMDA receptors (Omkumar et al., 1996; Raman et al., 1996; Leonard and Hell, 1997; Tingley et al., 1997), and on class C L-type Ca\(^{2+}\) channels (Hell, unpublished results; see also De Jongh et al., 1996). In this context it is interesting that protein–protein interactions between PKA or PKC with proteins known as A-kinase anchoring proteins (AKAPs) are necessary for the tonic upregulation of AMPA-type glutamate receptors by constitutive phosphorylation by PKA (Rosenmund et al., 1994). Similar to NMDA receptors, the activity of AMPA receptors is upregulated in hippocampal cells by PKA (Greengard et al., 1991; Blackstone et al., 1994) as well as by PKC (Wang et al., 1994a) and CaMKII (McGlade-McCulloh et al., 1993). Because interactions between PKA and AKAPs in muscle cells is also important for PKA-mediated potentiation of skeletal muscle L-type Ca\(^{2+}\) channels (Johnson et al., 1994, 1997), it is possible that PKA is localized next to postsynaptic class C L-type Ca\(^{2+}\) channels by a postsynaptic AKAP. AKAP 79 and AKAP 150 are potential candidates as they are associated with postsynaptic structures (Carr et al., 1992). Interestingly, AKAP 79 can also bind to PKC (Kluck et al., 1996) and to the Ca\(^{2+}\) dependent phosphatase 2B or calcineurin (Coghlan et al., 1995), thereby serving as a scaffold protein for these enzymes at locations crucial for signaling through serine/threonine phosphorylation (Faux and Scott, 1996).

Finally, phosphorylation of some sites of the ion channels discussed above may disrupt rather than foster the interaction with structural proteins such as the SAPs. Accordingly, it has been shown recently that PKA-mediated phosphorylation of the K\(^{+}\) channel Kir2.3 prevents this channel from binding to PSD-95/SAP90 in vitro (Cohen et al., 1996). Such a disruption may be necessary to allow reconstruction of synapse when they undergo long-lasting plastic changes such as long-term potentiation (LTP) (Bear and Malenka, 1994), which requires the activity of serine/threonine protein kinases (Madison et al., 1991) and of PTKs (Grant et al., 1992).
CONCLUSION

Phosphorylation of specific amino acids of postsynaptic ion channels such as AChR, NMDA receptors or class C L-type Ca\(^{2+}\) channels may control both the subcellular localization and the activity of these ion channels. Conversely, binding to structural proteins may change the activity of ion channels either directly or indirectly by inducing further phosphorylation by PTKs or serine/threonine kinases. Data supporting these models are very limited. However, methods to study the interactions between postsynaptic ion channels and structural proteins are now available and the proposed models will be tested within the next few years.

Acknowledgements—The author would like to thank Dr Peter Lipton, University of Wisconsin at Madison, for critically reading the manuscript and Dr Stanley C. Froehner, University of North Carolina at Chapel Hill, for sharing unpublished results. This work was supported in part by Faculty Scholar Award FSA-94-033 from the Alzheimer’s Association.

REFERENCES


Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995) PTB domain binding to signaling proteins through a


