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Long-term potentiation and synaptic protein phosphorylation

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Abstract

Long-term potentiation (LTP) is a well known experimental model for studying the activity-dependent enhancement of synaptic plasticity, and because of its long duration and its associative properties, it has been proposed as a system to investigate the molecular mechanisms of memory formation. At present, there are several lines of evidence that indicate that pre- and postsynaptic kinases and their specific substrates are involved in molecular mechanisms underlying LTP. Many studies focus on the involvement of protein kinase C (PKC). One way to investigate the role of PKC in long-term potentiation is to determine the degree of phosphorylation of its substrates after in situ phosphorylation in hippocampal slices. Two possible targets are the presynaptic membrane-associated protein B-50 (a.k.a. GAP 43, neuromodulin and F1), which has been implicated in different forms of synaptical plasticity in the brain such as neurite outgrowth, hippocampal LTP and neurotransmitter release, and the postsynaptic protein neurogranin (a.k.a. RC3, BICKS and p17) which function remains to be determined. This review will focus on the protein kinase C activity in pre- and postsynaptic compartment during the early phase of LTP and the possible involvement of its substrates B-50 and neurogranin.

Key words: Long-term potentiation; Protein phosphorylation; B-50; Neurogranin; Protein kinase C

1. Introduction

When brain biochemistry was the science of documenting the chemical content of neural tissue, the abundance of phosphors in the brain led to simplified speculations such as 'Ohne Phosphorus, keine Gedenken'. It took more then a century to demonstrate the significance of cyclic phosphorylation and dephosphorylation reactions in brain function. In the 1960's the work of Heald and later that of Rodnight and Greengard revealed an intimate relationship between neuronal depolarization and plasma membrane protein phosphorylation. At present, a multifold of specific protein kinases and phosphatases are involved in synaptic events such as transmitter release and receptor activation (desensitization, ion channel dynamics, etc). Protein phosphorylation is also implicated in processes underlying synaptic plasticity such as long-term potentiation (LTP). Although there is still much discussion on the role of pre- vs. postsynaptic mechanisms in the induction and maintenance of LTP, there is growing evidence that specific pre- and postsynaptic protein kinases and substrate proteins play a role in different aspects of LTP. Straight forward classical neurochemical techniques allow proper evaluation of especially presynaptic mechanisms (using presynaptic preparations such as synaptosomes) whereas due to the small dimensions of the presynaptic terminal, electrophysiologists are limited to record from the large postsynaptic cell bodies. It is expected that in the near future experiments will be conducted that simultaneously measure chemical and electrical mechanisms in LTP at single cell level. In the present paper we review some of the literature concerning two protein kinase C (PKC) substrates and pre- and postsynaptic PKC in the maintenance of LTP.

1.1. B-50/GAP-43

B-50 (also known as growth-associated protein-43 (GAP-43), neuromodulin and F1; reviewed by: Liu and Storm [45]; Gispen et al. [33]; Strittmatter et al. [59]) is a well documented PKC substrate found predominantly in neurons at the cytosolic site of the presynaptic and the growth cone membrane. cDNA sequence analysis revealed rat B-50 as a 23.6-kDa protein of 226 amino acids with

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an apparent molecular weight of 43–48 kDa on SDS-PAGE. Palmitoylation of Cys-3 and/or Cys-4 of B-50 is important for its plasma membrane-association and is not required for its phosphorylation by PKC. In vitro, B-50 has been reported to bind calmodulin and actin, to serve as a substrate for several kinases and phosphatases, to affect polyphosphoinositol metabolism by inhibiting phosphatidylinositol 4-phosphate (PIP) kinase, and to augment the binding of GTP-γ-S to hetrotrimeric GTP-binding proteins G_o and G_i (reviewed by Liu and Storm [45]; Strittmatter et al. [59]; De Graan and Gispen [24]). The calmodulin-binding properties of B-50 differ from most other known calmodulin-binding proteins, because B-50 binds calmodulin in the absence of Ca^{2+} and releases calmodulin in the presence of Ca^{2+}. The phosphorylation state of B-50 may be a regulatory factor in calmodulin binding, because phosphorylation of Ser-41 within the presumed calmodulin-binding domain (residues 39–51), reduces the affinity of B-50 for calmodulin. In vitro, phosphorylation of B-50 by casein kinase II (presumably at Ser-192 and/or Ser-193) did not affect the ability of B-50 to bind calmodulin, whereas calmodulin binding to B-50 inhibited casein kinase II-mediated phosphorylation of B-50 [6]. This indicates that calmodulin regulates casein kinase II-mediated B-50 phosphorylation by binding to B-50 and thus sterically hinders the interaction of casein kinase II with its phosphorylation sites on B-50. Because PKC-mediated B-50 phosphorylation prevents calmodulin binding to B-50, casein kinase II-mediated B-50 phosphorylation may indirectly be controlled by PKC-mediated B-50 phosphorylation. Finally, dephosphorylation of Ser-41 in B-50 can be mediated by type 1 and type 2A protein phosphatases present in presynaptic membranes and in vitro by Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin [34,44,58].

B-50 has been implicated in the modulation of different forms of synaptical plasticity in the brain, including neurite outgrowth and regeneration, hippocampal long-term potentiation and neurotransmitter release (reviewed by: Liu and Storm [45]; Strittmatter et al. [59], De Graan and Gispen [24]). At a molecular level, B-50 regulates these processes by modulating signal transduction pathways in growth cones and mature nerve terminals. Indeed, the degree of PKC-mediated B-50 phosphorylation has been correlated with plastic synaptic alterations in hippocampal neurons [17], enhanced neurotransmitter release from hippocampal slices and synaptosomes [26,28], inhibition of the PIP kinase activity [61] and more recently with growth cone mobility [31,64] and long-term potentiation in hippocampal slices [32,53]. Thus, it seems that PKC-mediated B-50 phosphorylation is of major physiological importance. Indeed, we observed that antibodies, which specifically inhibited PKC-mediated B-50 phosphorylation, inhibited Ca^{2+}-induced noradrenaline and neuropeptide cholecystokinin-8 release from permeated synaptosomes, whereas anti-B-50 antibodies, which did not affect B-50 phosphorylation, were without effect [24]. These data confirm the causal relation between B-50 and neurotransmitter release and suggest that B-50 regulates neurotransmitter release in a phosphorylation-dependent manner. Developmental studies, in which B-50 was depleted from primary sensory neurons [2] or overexpressed in PC12 cells [52] and in which phosphorylation-site mutated B-50 was transfected in non-neuronal cells [64], indicated that B-50 modulates growth cone formation and activity in a phosphorylation-dependent manner, rather than being required for neurite outgrowth per se [7]. In vivo, it was demonstrated that PKC-phosphorylated B-50 is only present in the nerve terminals, and not along the axon and in the cell body, and during axonogenesis only in the distal axon and growth cone [49]. Altogether these data might suggest that PKC only acts on B-50 in regions where membrane is being actively added and that B-50 might fulfil a common role in neuronal plasticity during development and adulthood.

1.2. Neurogranin

Neurogranin, also known as RC3 [62] and B-50 immunoreactive C-kinase substrate (BICKS) [19] is a second neuro-specific substrate of PKC [11,12], mostly expressed in forebrain neurons during postnatal development and maturity, and localized predominantly in dendritic spines [54,63]. This postsynaptic PKC substrate, purified to homogeneity from bovine forebrain, consists of 78 amino acid residues, has a molecular mass of 7.8 kDa and is a member of the family of acidic substrates of PKC (*p*I 5.6) [12,38].

Neurogranin and B-50 have several properties in common. They share a highly conserved domain of 18 amino acids, which contains the unique PKC phosphorylation site [6,12,38] and the atypical calmodulin-binding domain. As B-50, neurogranin binds calmodulin at low calcium concentrations [12,19,38]. Although different in sequence and molecular mass, neurogranin and B-50 display the same anomalous migration behavior on SDS-PAGE, depending on the percentage of polyacrylamide [12,58]. Both proteins are soluble in 2.5% perchloric acid [11]. In contrast to the presynaptic localization of B-50, neurogranin is abundant in dendrites and is localized in dendritic shafts and spines [54,63]. The two proteins also differ in their cellular localization: neurogranin is found in the cytosol whereas B-50 is predominantly membrane-associated.

The physiological role and the functional consequences of neurogranin phosphorylation remain to be elucidated. Recently, Watson and coworkers proposed that PKC

phosphorylated neurogranin may play a role in calcium homeostasis in dendrites of forebrain neurons by modulating the inositol 1,4,5-triphosphate and diacylglycerol second messenger pathways [20]. Alternatively, neurogranin may serve as local calmodulin store, releasing calmodulin in response to PKC-mediated phosphorylation and a rise in intracellular calcium levels, thus activating postsynaptic Ca^{2+}/calmodulin dependent signal transduction pathways.

1.3. LTP and PKC

Many studies concerning the involvement of phosphorylation processes in LTP focus on the role of PKC and here we will shortly review some of these studies (for a detailed review see Colley and Routtenberg [21].

One way to study the role of PKC in LTP is to determine the effect of bath application of membrane permeable protein kinase inhibitors on the maintenance of LTP. In most of these studies no distinction is made between pre- and postsynaptic PKC. Among the inhibitors tested H-7, sphingosine, polymyxin B (PMB) and K-252b appear to suppress LTP [39,46,55]. These results indicate that PKC plays an important role in LTP and are in line with data showing a translocation of PKC activity and immunoreactivity following tetanic stimulation [3,43]. A significant problem associated with this pharmacological approach is the limited specificity of the inhibitors used towards other kinases and cellular physiology. For example Malinow et al. [47] found that application of H-7 after the induction of LTP caused a return to baseline levels of potentiated EPSPs without affecting non-potentiated EPSPs, whereas Muller et al. [50], using the same techniques, found that potentiated as well as unpotentiated EPSPs were reduced.

More specific information about the involvement of (postsynaptic) PKC in LTP was obtained by injecting PKC [36] or kinase inhibitors directly into the postsynaptic cell. Intracellular injection of PKC(19–31), a peptide fragment representing the pseudosubstrate region of the regulatory domain of PKC, prevented the maintenance of LTP in the injected cell whereas the neighbouring cells showed normal LTP [48]. These results indicate that postsynaptic PKC is involved in the maintenance of LTP. A combination of bath application and intracellular injection of inhibitors was performed by Huang et al. [37]. They found that postsynaptic injection of H-7 or PMB only affected LTP maintenance in the Schaffer collateral–CA1 pathway, when injected 10 min before conditioning, but not when injected 5 min after conditioning. Extracellular application of H-7 or PMB in the stratum radiatum of the CA1 region 15 or 30 min after conditioning of the pathway also caused a decrease of the potentiated EPSPs to baseline levels. Interestingly, extracellular application of H-7 or PMB 60 min after conditioning did not affect the potentiated EPSPs. Thus, it appears that there is a transient involvement of PKC in the maintenance of LTP. Which PKC isozyme is involved remains to be determined. Recently is was shown that in hippocampal slices from γ-PKC mutant mice LTP was strongly diminished [1].

One possible postsynaptic target for PKC modulation are the NMDA currents [13]. Indeed there is evidence suggesting that NMDA receptors can be regulated by PKC-mediated phosphorylation. For example, the potentiation of NMDA currents in *Xenopus* oocytes by metabotropic glutamate receptors is mediated by PKC [40] and PKC activation reduces the Mg^{2+} block of the NMDA receptor in isolated trigeminal neurons [18]. Another possible target for postsynaptic PKC is neurogranin. Recent post-hoc phosphorylation studies using neurogranin peptide substrates, show increased PKC activity after LTP induction [41,42].

Several early studies have used post-hoc phosphorylation of substrate proteins to investigate the molecular mechanisms underlying LTP. The first report [14] shows that conditioning of the Schaffer collateral–CA1 pathway in hippocampal slices induces changes in phosphate incorporation in three proteins, with molecular weights of 53, 40 and 27 kDa, respectively. Two of these proteins showed an increased phosphate incorporation in a post-hoc phosphorylation assay (namely a 53 and 27kDa protein), whereas a 40 kDa protein showed a decrease incorporation in this assay. This 40 kDa protein was identified as the α-subunit of pyruvate dehydrogenase (α-PDH), a major phosphoprotein in the mitochondria [15]. The change in α-PDH phosphorylation is only short-lasting, thus implicating a role in short term changes after tetanic stimulation [16]. We showed that tetanic stimulation of the same pathway in hippocampal slices resulted in an increased post-hoc phosphorylation of a 52 kDa protein [8]. In subsequent studies we established that this change in 52 kDa phosphorylation and that in the evoked field potentials were strongly correlated [60]. The 52 kDa protein was present in the synaptosomal plasma membrane fraction and its in vitro phosphorylation did not depend on cAMP or Ca^{2+}/Calmodulin [9]. This 52 kDa protein was identified as the coated vesicle phosphoprotein pp50 [57].

Tetanic stimulation also increased the post-hoc phosphorylation of a 48 kDa phosphoprotein which was identified as B-50. This increase in B-50 phosphorylation after tetanic stimulation in hippocampal slices was much less consistent than that found for the 52 kDa protein [8]. But after induction of LTP in vivo Routtenberg et al. [56] observed an increase in the post-hoc phosphorylation of

F1 (B-50), which was closely correlated with the changes in the evoked population spike.

As mentioned, this increase in B-50 phosphorylation after LTP induction were determined using a post-hoc phosphorylation assay. This post-hoc phosphorylation assay has some interpretation problems and therefore a new method of quantitative immunoprecipitation was developed to determine the in situ phosphorylation of B-50 in the hippocampal slice [22]. Using this assay we have performed a series of experiments with the following experimental design: three hippocampal slices (450 μm) were placed in a recording chamber, in two slices stimulation and recording electrodes were placed, hereafter slices were labelled with 100 μCi/ml [^{32}P]orthophosphate for 90 min. One of the slices received a high frequency train of pulses (100 Hz, 1 s, test intensity), whereafter experiments were continued for 10, 30, 60, 90 or 120 min, the electrodes were removed, and the slices were homogenized. B-50 phosphorylation in these slices was then determined after quantitative immunoprecipitation [22].

These experiments [32,53] have shown that 60 min after induction of LTP in the Schaffer collateral—CA1 pathway in the hippocampal slice, there is a significant increase in the phosphorylation of B-50. This increase in B-50 phosphorylation was strongly correlated with the increase in the evoked field potentials, could be prevented by application of the NMDA-receptor antagonist APV (which also blocks the induction of LTP), and only occurred when high-frequency stimulation results in expression of LTP (in a few cases tetanic stimulation failed to induce LTP and in these cases there was no change in B-50 phosphorylation). A significant increase in B-50 phosphorylation could already be detected 10 min after the tetanus (the earliest timepoint measured) and lasted at least 60 min. At 90 min and 2 h after LTP induction no difference in B-50 phosphorylation could be detected anymore between slices showing LTP and slices only receiving low frequency stimulation. As mentioned before, B-50 is localized in the presynaptic terminal whereas neurogranin, which is identical to B-50 in its unique PKC phosphorylation site and its calmodulin binding domain, is localized postsynaptically. Because it is still a matter of debate whether LTP expression is a presynaptic or postsynaptic phenomenon (or both), we have modified the B-50 immunoprecipitation procedure to enable concomitant determination of the phosphorylation state of B-50 (presynaptic) and neurogranin (postsynaptic) in the same slice [53]. Using this protocol we found that neurogranin phosphorylation also increases after LTP induction [25] (Ramakers et al., in preparation). The time course of neurogranin phosphorylation after tetanic stimulation, however, differs from that of B-50 phosphorylation, neurogranin only shows a significant increase in phosphorylation 60 min after LTP induction. The increase in neurogranin phosphorylation could be prevented by the NMDA-receptor antagonist APV. In slices in which high frequency stimulation failed to induce LTP, there was neither a change in B-50 nor in neurogranin phosphorylation 60 min after high-frequency stimulation. These findings indicate that the increases in B-50 and neurogranin phosphorylation are dependent on the expression of LTP, and are not caused by repetitive stimulation of afferent fibres. The increase in B-50 and neurogranin phosphorylation is highly correlated with the increase in the evoked responses (EPSPs) at each timepoint. Our results show that after the induction of LTP both pre- and postsynaptic PKC-mediated phosphorylation increases, but in different time windows.

2. Discussion

In the preceding paragraphs we discussed the significance of PKC activity in pre- and postsynaptic compartments during the early phase of LTP. We have studied this PKC activity by monitoring the degree of phosphorylation of two identified PKC substrates, B-50 as presynaptic marker, neurogranin as postsynaptic marker. Although certainly not unique, B-50 and neurogranin are important PKC-substrate proteins, the phosphorylation of which is influenced by tetanic stimulated leading to LTP. Blockade of the NMDA receptor by APV (thus blocking the onset of LTP), low-frequency stimulation or unsuccessful tetanic stimulation, do not induce parallel changes in B-50, or neurogranin phosphorylation. Notwithstanding the relatively short period following the induction of LTP that these changes in B-50 or neurogranin phosphorylation occur, their specificity to the phenomenon of LTP suggests that they are not merely an experimental epiphenomenon. It remains to be shown whether the increase in the phosphorylation state of B-50 and neurogranin are consequences of the induction of LTP and whether they are of significance to the mechanisms underlying the early phase of LTP.

Even if we assume that the changes in the phosphorylation state of B-50 and neurogranin are causally related to LTP, it is still difficult to assess what the meaning of these molecular correlates of LTP is. In fact, there is considerable uncertainty about the role of B-50 and neurogranin in neural signal transduction and plasticity. In Fig. 1 we depict a working hypothesis—one out of many possible—that takes into account that both proteins are atypical calmodulin binding proteins, are PKC substrates and have a distinct synaptic localization. In the presynaptic compartment, the depolarization-induced influx of Ca^{2+} and the activation of PKC results in a net change in the degree of B-50 phosphorylation and the release of

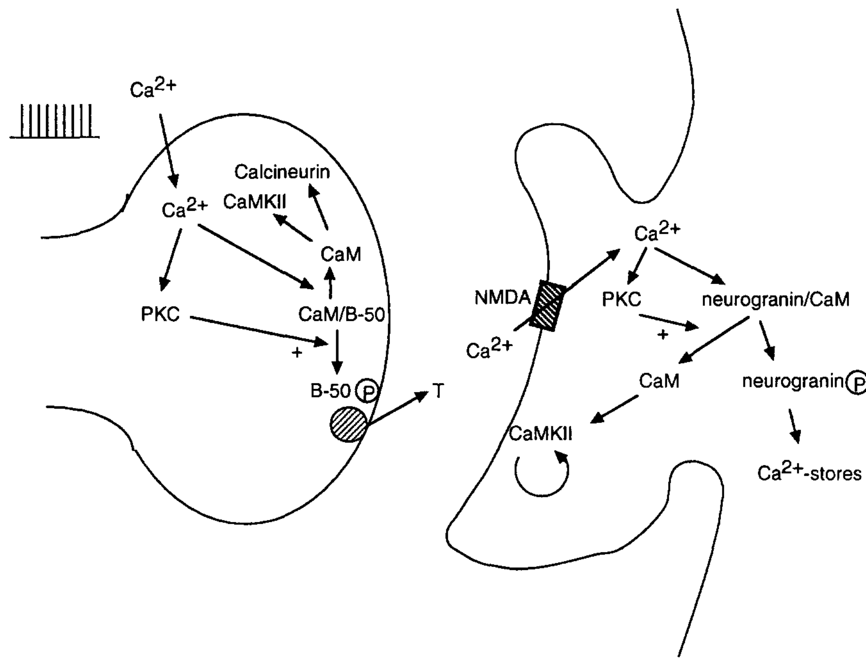

Fig. 1. The possible roles of B-50 and neurogranin in LTP. At the presynaptic terminal an influx of Ca^{2+}-ions (through voltage-activated Ca^{2+}-channels) causes a dissociation of the calmodulin/B-50 complex and an activation of PKC. This activated PKC can phosphorylate the dissociated B-50 which in turn is involved in the regulation of neurotransmitter release. The dissociated calmodulin activates Ca^{2+}/calmodulin sensitive enzymes (e.g. the Ca^{2+}/calmodulin-dependent protein kinase II or calcineurin). At the postsynaptic site Ca^{2+}-influx through NMDA-receptors, which are activated during high frequency stimulation, leads to a dissociation of the calmodulin/neurogranin complex and an activation of PKC. Neurogranin can then be phosphorylated by the activated PKC and release Ca^{2+} from internal stores. Calmodulin can again activate Ca^{2+}/calmodulin sensitive enzymes (as in the presynaptic terminal), which could play a role in LTP.

B-50-bound calmodulin. The size of B-50-bound calmodulin pool depends on the degree of phosphorylation of B-50, because phosphorylation and calmodulin binding are mutually exclusive. The increase in free calmodulin might locally regulate the activity of Ca^{2+}/calmodulin sensitive enzymes such as Ca^{2+}/calmodulin-dependent kinase II and calcineurin. From other studies it is conceivable that these changes facilitate neurotransmitter release. In fact some, not all, reports have pointed to enhanced glutamate-release during the early phase of LTP. It may be that the enhanced phosphorylation of the presynaptic protein B-50 is a reflection of that process. Alternatively, the changes in B-50 phosphorylation could be related to the dynamics in presynaptic terminal morphology as described following hippocampal LTP [10], because B-50 has been implicated in the mechanism of neurite outgrowth and in growth cone function.

In the postsynaptic compartment, neurogranin may serve as a local calmodulin sink. As described for B-50, increased Ca^{2+} influx and PKC activation yields enhanced neurogranin phosphorylation and thus a local increases in free calmodulin. This calmodulin may subsequently activate Ca^{2+}/calmodulin sensitive enzymes regulating signal-transduction pathways involved in the maintenance of LTP. On the other hand, like phosphorylated B-50, phosphorylated neurogranin itself may also be of significance to the maintenance of LTP. In this respect the suggestion that phosphorylated neurogranin may be involved in regulating the releases Ca^{2+} from intracellular stores [20] is worth considering.

In summary the data reviewed are consistent with activation of pre-as well as postsynaptic PKC following tetanic stimulation. It remains to be determined whether different PKC isozymes are causally involved in the maintenance of LTP or rather play a modulatory role [1]. These PKC isozymes most likely have multiple substrates, with distinct synaptic localizations. It will be important to establish which PKC isozymes with their specific substrates are important during LTP. Because B-50 and neurogranin are identified PKC substrates, which show increased in situ phosphorylation after tetanic stimulation, further research will be focused on the elucidation of the physiological role of these proteins in neuronal signal-transduction.

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