The role of B-50/GAP-43 in transmitter release: Studies with permeated synaptosomes

Pierre N. E. De Graan* and Willem-Hendrik Gispen
Division of Molecular Neurobiology, Rudolf Magnus Institute, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Introduction

Depolarization of the presynaptic terminal stimulates Ca^{2+} influx and exocytosis of neurotransmitters. In synaptosomes the influx of Ca^{2+} activates Ca^{2+}-dependent protein kinases and phosphatases, resulting in changes in the degree of phosphorylation of a variety of proteins. These changes in protein phosphorylation have been implicated in the molecular mechanism of neurotransmitter release. The major phosphorylation systems thought to be involved in transmitter release are Ca^{2+}/calmodulin (CaM)-dependent kinase type II phosphorylating synapsin I [1, 2], and the Ca^{2+}/phospholipid-dependent protein kinase C (PKC) phosphorylating the actin filament cross-linking myristoylated alanine-rich C kinase substrate (MARCKS) [3, 4] and B-50/GAP-43 [5]. In this paper we focus on the role of the presynaptic PKC substrate B-50/GAP-43 in the mechanism of transmitter release.

Protein B-50 (also known as GAP-43, pp46, neuromodulin and F1) is a nervous tissue-specific protein, which is highly expressed in neurons during development and nerve regeneration, and has been implicated in neurite outgrowth, long-term potentiation, signal transduction and neurotransmitter release (for reviews, see [6–12]). In mature neurons B-50 is expressed in most (if not all) cases. It is found predominantly in presynaptic membranes and not in dendrites [13, 14].

Rat B-50 is a 226-amino-acid membrane-associated protein, which is attached to the presynaptic membrane through two cysteine residues at positions 3 and 4, either by thioester linkage to palmitic acid [15] or by some other, still unresolved mechanism [16, 17]. B-50 is a well characterized PKC substrate with a single PKC phosphorylation site at Ser-41. B-50 can be dephosphorylated by type 1 and type 2A protein phosphatases present in synaptic plasma membranes (SPM) [18, 19] and *in vitro* by type 2B protein phosphatases [20, 21], a Ca^{2+}/CaM-dependent phosphatase family, known as calcineurins (CaN). B-50 is an atypical CaM-binding protein because it has a higher affinity for CaM in the absence than in the presence of Ca^{2+} [22, 23]. The phosphorylation state of B-50 may be a regulatory factor in CaM binding, as phosphorylation of Ser-41 within the presumed CaM-binding domain (residues 39–51) reduces the affinity of B-50 for CaM [24].

On the basis of its presynaptic localization, its proposed role in signal transduction, and the high degree of correlation between the degree of PKC-mediated B-50 phosphorylation and neurotransmitter release, we have proposed that B-50 is involved in the molecular mechanism of neurotransmitter release. A major problem in studying the mechanism of Ca^{2+}-induced transmitter release and the role of B-50 therein, is the inaccessibility of the interior of the presynaptic terminal to experimental manipulations. The small size of most terminals precludes microinjection and electrophysiological approaches. Therefore, we developed a method of permeating purified synaptosomes using the bacterial toxin streptolysin-O (SL-O) [25], which does not affect the exocytotic machinery. This permeated synaptosome preparation enables us to manipulate the intrasynaptosomal ionic conditions and to introduce membrane-impermeable macromolecules, which influence presynaptic signal transduction.

In this paper we summarize the properties of the permeated synaptosome preparation and the use of this preparation to investigate the role of B-50 in Ca^{2+}-induced release of noradrenaline (NA) and the neuropeptide cholecystokinin-8 (CCK-8).

Ca^{2+}-induced NA release from permeated synaptosomes

To enable more detailed analysis of the mechanisms underlying transmitter release, we studied Ca^{2+}-induced NA release (using h.p.l.c. techniques or [^{3}H]NA) from highly purified cerebrocortical synaptosomes which were permeated with SL-O [10, 25–27]. Under optimal permeation conditions (0.3 IU/ml SL-O), elevation of the free Ca^{2+} concentration in the permeation buffer from 10^{-8} to

Abbreviations used: CaM, calmodulin; CaN, calcineurin; CCK, cholecystokinin; LDH, lactate dehydrogenase; NA, noradrenaline; PKC, protein kinase C; PP, protein phosphatase; SL-O, streptolysin-O; SPM, synaptic plasma membrane; TeTx, tetanus toxin.

*To whom correspondence should be addressed.

10^{-5}M induced a NA release amounting to 12.9 ± 1.3% ($n = 5$) of the total [^{3}H]NA incorporated. Basal [^{3}H]NA release (release at 10^{-8}M Ca^{2+}) at 37°C was 34.7 ± 1.0% ($n = 5$) of the total [^{3}H]NA incorporated. Basal [^{3}H]NA release could be reduced considerably (to 17.2 ± 2.3%) by lowering the incubation temperature to 25°C. Ca^{2+}-induced endogenous NA release amounted to 12% of the total endogenous NA content of 8.9 pmol NA/mg synaptosomal protein. No difference was observed in Ca^{2+} sensitivity of the release from ^{3}H-labelled and endogenous NA pools. Significant Ca^{2+}-induced NA release was observed after raising the Ca^{2+} concentration from 10^{-8} to 10^{-6}M. Half-maximal release was observed at about 3×10^{-6}M and maximal release between 10^{-5}M and 10^{-4}M Ca^{2+}.

Ca^{2+}-induced [^{3}H]NA release was largely (>60%) ATP dependent. In the absence of exogenous ATP, Ca^{2+}-induced [^{3}H]NA release was about 5% and basal NA release was rather high (47%). Addition of exogenous ATP strongly reduced basal [^{3}H]NA release and, to a lesser extent, NA release in the presence of 10^{-5}M Ca^{2+}. Optimal Ca^{2+}-induced NA release was achieved at about 2 mM ATP, which is close to the physiological concentration (see [28]). The effect of ATP on basal NA release could signify the ATP requirement for maintenance of the proton gradient over the vesicular membrane required for sequestration of catecholamines in synaptic vesicles [28].

To confirm the vesicular nature of the Ca^{2+}-induced NA release, we studied the efflux of lactate dehydrogenase (LDH) and the effect of tetanus toxin (TeTx) on Ca^{2+}-induced release. Indeed, the efflux of the cytosolic marker protein LDH was independent of the free Ca^{2+} concentration. Intact TeTx is known to inhibit Ca^{2+}-induced vesicular neurotransmitter release, probably by proteolytic cleavage of the vesicle-associated protein synaptobrevin through the action of its light chain. In SL-O-permeated synaptosomes, we have shown recently that TeTx light chain inhibits Ca^{2+}-induced NA release concentration-dependently [29], which is consistent with vesicular NA release in this system.

Ca^{2+}-induced CCK-8 release from permeated synaptosomes

The neuropeptide CCK is one of the most abundant endogenous peptidergic transmitters in the brain [30]. In rat cerebral cortex it is released predominantly as a sulphated octapeptide in a depolarization-induced and Ca^{2+}-dependent manner. CCK-8 is stored in large dense-cored vesicles, is thought to be released outside the active zone and is often co-localized with classic transmitters. The trigger for CCK-8 release is thought to be $[Ca^{2+}]_i$ [31].

We studied the Ca^{2+} dependency of the CCK-8 release from SL-O-permeated synaptosomes [32]. Ca^{2+}-induced CCK-8 release was maximal at SL-O concentrations above 0.3 IU/ml, as was observed for Ca^{2+}-induced NA release from SL-O-permeated synaptosomes [10]. Increasing the degree of permeation hardly increased CCK-8 release under basal, 10^{-8}M Ca^{2+} conditions. This confirms the vesicular nature of Ca^{2+}-induced CCK-8 release from SL-O-permeated synaptosomes and indicates that CCK-8-containing large dense-cored vesicles are much less sensitive to SL-O treatment than NA-containing vesicles [10, 32]. Our data suggest that CCK-8 release is initiated at a slightly higher Ca^{2+} concentration (3×10^{-6}M) than observed for NA (10^{-6}M) [32], but we cannot rule out that this is due to a difference in the sensitivity of the detection assays for the transmitters. Maximal Ca^{2+}-induced release for CCK-8 as well as for NA was observed at about 10^{-4}M Ca^{2+}. These high Ca^{2+} concentrations may occur near sites of exocytosis and are demonstrated in presynaptic terminals of the giant squid [33]. However, it cannot be excluded that the high Ca^{2+} concentrations are required because of a decrease in Ca^{2+} sensitivity of the release machinery as a result of leakage of Ca^{2+}-dependent proteins from the synaptosomal interior after permeation, as has been shown, for instance, for permeated chromaffin cells [34].

In contrast to Ca^{2+}-induced NA release from permeated synaptosomes, which is largely (>60%) ATP-dependent [26, 27], Ca^{2+}-induced CCK-8 release was only partially (30%) dependent on the presence of exogenous ATP. These observations are in accordance with the fact that peptide-containing vesicles are formed in the cell body and are not, like catecholamine-containing vesicles, dependent on ATPase-mediated vesicle storage in the nerve terminal itself. The partial ATP dependence may indicate that kinase-mediated phosphorylation events are not important in steps in the mechanism of large dense-cored vesicle fusion occurring after the Ca^{2+} influx. Of course, neither the involvement of kinases before the Ca^{2+} trigger, nor the involvement of phosphatases can be excluded. To obtain specific information on the role of phosphorylation reactions, we studied the involvement of PKC and its presynaptic substrate B-50 in Ca^{2+}-induced NA and CCK-8 release.

PKC-mediated B-50/GAP-43 phosphorylation and release

To study the involvement of PKC in Ca^{2+}-induced NA and CCK-8 release, we tested the effect of the synthetic peptide PKC_{19-36}, which corresponds to the autoinhibitory sequence of PKC. Endogenous B-50 phosphorylation in SPM and permeated synaptosomes are potently inhibited by PKC_{19-36} [27]. PKC_{19-36} tested up to 10^{-5}M did not affect either Ca^{2+}-induced [^{3}H]NA or CCK-8 release from permeated synaptosomes [27]. In addition, evidence from other experiments [27] suggests that PKC is not involved in steps in the release process following the Ca^{2+} trigger. Firstly, phorbol ester-induced PKC down-regulation in synaptosomes did not affect Ca^{2+}-induced release. Secondly, Ca^{2+}- and phorbol ester-induced release were found to be additive. Of course, our data do not rule out an involvement of PKC before the Ca^{2+} trigger. In fact, in earlier studies we found a close correlation between PKC-mediated B-50 phosphorylation and neurotransmitter release (for review see [10]).

To investigate the role of presynaptic PKC substrate B-50 in neurotransmitter release, we introduced poly- and monoclonal anti-B-50 IgGs into permeated synaptosomes [25–27, 32, 35]. Affinity-purified polyclonal anti-B-50 IgGs, which inhibit B-50 phosphorylation, inhibited Ca^{2+}-induced [^{3}H]NA release dose-dependently [25–27]. Two different monoclonal anti-B-50 IgGs, NM2 and NM6 [36], were used. NM2 recognizes an epitope located within the proposed CaM-binding domain of B-50 (amino acids 39–51), containing the only PKC phosphorylation site at Ser-41 and inhibits B-50 phosphorylation. NM6, recognizing a C-terminal-located epitope at B-50 domain 132 to 213, does not interfere with PKC-mediated B-50 phosphorylation. Both monoclonals recognized B-50 specifically on Western blots loaded with synaptosomal proteins, and cross-reacted with phospho- as well as dephospho-B-50. The affinity of anti-B-50 IgGs for B-50 differed when tests were carried out in a solid-phase e.l.i.s.a. The titre (=[IgG protein] at half-maximal binding) of monoclonal NM6 for CaM-Sepharose-purified rat B-50 was about 10-fold higher, and, for B-50 present in lysed synaptosomes, at least threefold higher than the titre of monoclonal NM2.

Anti-B-50 IgGs NM2 inhibited Ca^{2+}-induced [^{3}H]NA, endogenous NA and CCK-8 release [32, 35]. Half-maximal inhibition of Ca^{2+}-induced [^{3}H]NA release was observed at 1.5 μg NM2 IgG/60 μl ($P<0.05$) and maximal inhibition at 3.0 μg NM2 IgG/60 μl. At this concentration (3.0 μg IgG/60 μl) NM6 affected neither Ca^{2+}-induced [^{3}H]NA and endogenous NA release nor CCK-8 release. Only concentrations ≥ 6.0 μg/60 μl NM6 slightly inhibited Ca^{2+}-induced [^{3}H]NA release. The LDH efflux was not affected by the presence of either anti-B-50 IgGs NM2 or NM6 (at 3 μg IgG/60 μl), indicating that the inhibitory effect of the anti-B-50 IgGs was not due to a decrease in permeation efficiency. The fact that N-terminal-directed NM2 inhibits release, whereas C-terminal-directed NM6 is without effect, shows that the inhibition by the antibody is not due to non-specific steric hindrance by the antibodies. In summary, these data provide strong evidence for a role of the N-terminus of B-50 in the mechanism of Ca^{2+}-induced neurotransmitter release from large dense-cored vesicles.

Concluding remarks

Our antibody interference experiments show that the N-terminus of B-50 is important for release. The N-terminal domain of B-50 contains the membrane targeting signal, the CaM-binding domain and the PKC phosphorylation site. Because most of the B-50 in synaptosomes is membrane-bound, it is unlikely that the antibodies affect membrane attachment. Initially we thought that PKC-mediated B-50 phosphorylation was essential for NA release. This interpretation was based on the close correlation between the degree of B-50 phosphorylation and transmitter release (for review see [10]) and the fact that anti-B-50 IgGs, which inhibit B-50 phosphorylation, interfere with NA release from permeated synaptosomes [25]. However, more recently we provided substantial evidence against a role of PKC in steps in release after the Ca^{2+} trigger (see above). Thus, it appears that the phosphorylation state of B-50, rather than its PKC-mediated phosphorylation, is important for steps after the Ca^{2+} trigger. This prompted us to study two other biochemical properties of B-50, confined to the N-terminal domain, which co-determine the degree of B-50 phosphorylation: B-50 dephosphorylation and the binding of CaM to B-50.

Preliminary experiments show that monoclonal NM2 inhibits B-50 dephosphorylation by endogenous synaptosomal phosphatases, whereas N-terminal-directed NM6 is without effect [35]. In our synaptosomal preparation phosphatase activity was inhibited by about 80% by 1 μM okadaic acid, a potent and specific inhibitor of protein phosphatase (PP)-1 and PP-2A [37]. This is in accordance with reports that B-50 is mainly (90%) dephosphorylated by PP-1 and PP-2A in SPM [18, 19].

Although the physiological relevance of protein dephosphorylation in the process of neurotransmitter release remains to be established, several studies indicate that it may be important for release. In *Paramecium*, anti-CaN IgGs inhibited the dephosphorylation of a 65 kDa protein as well as exocytotic membrane fusion [38]. Interestingly, the Ca^{2+}/CaM-dependent protein phosphatase CaN (PP-2B) dephosphorylates B-50 *in vitro* [20, 21].

Another important N-terminal property of B-50 is its affinity for CaM under low Ca^{2+} conditions [22]. It has been suggested that B-50 serves as a local CaM store at the plasma membrane [8]. Although CaM itself may not cause vesicle fusion, several lines of evidence indicate that CaM might be involved in neurotransmitter release: pharmacological CaM inhibitors (reviewed in [39, 40]) and anti-CaM IgGs inhibit exocytosis [38, 40, 41]. One possibility is that CaM increases neurotransmitter release through activation of Ca^{2+}/CaM-dependent protein kinase II and subsequent synapsin I phosphorylation [1]. Indeed, preliminary experiments [35] show that NM2 inhibits [^{125}I]CaM binding to B-50. This observation is in line with the finding that [^{125}I]CaM overlay of SDS-denatured B-50 in PAGE-gels could be inhibited by polyclonal antibodies against B-50 fragment 43–51 [42].

In conclusion, using monoclonal anti-B-50 IgGs, we established a causal relationship between B-50 and Ca^{2+}-induced NA and CCK-8 release. Although a function of the C-terminal B-50 domain 132–226 cannot be excluded (e.g. B-50 phosphorylation by casein kinase II at serine residues 192 and 193, as observed in *in vitro* studies [43, 44], or binding to components of the cytoskeleton, as suggested to occur at the C-terminus of B-50 [45]), we demonstrated that the N-terminus of B-50 plays an important role in the mechanism of Ca^{2+}-induced NA and CCK-8 release. Which property at the N-terminus of B-50 is involved in the mechanism of Ca^{2+}-induced release from large dense-cored vesicles remains to be established, because the anti-B-50 IgGs NM2 do not distinguish between these distinct properties. It may well be that both the degree of B-50 phosphorylation and of CaM binding are important in the stimulus-secretion coupling, as B-50 phosphorylation and B-50–CaM binding are mutually exclusive. Moreover, CaM released from B-50 during a depolarization-induced Ca^{2+} influx might stimulate the rate of B-50 dephosphorylation directly by activating CaN. The SL-O-permeated synaptosome preparation offers unique possibilities for elucidating the role of CaM, B-50 dephosphorylation and protein phosphatases in the molecular mechanism of Ca^{2+}-induced NA and CCK-8 release. This preparation might also be used to study Ca^{2+}-induced release of glutamate.

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Glutamate release from isolated nerve terminals: Modulatory role of protein phosphorylation and dephosphorylation

Talvinder S. Sihra*
Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

Introduction

Three major events are elicited by an invading action potential arriving at the presynaptic nerve-terminal and these lead to the release of neurotransmitter by the exocytosis of synaptic vesicles [1]. Firstly, plasma membrane depolarization occurs transiently as a result of the collective activity of voltage-dependent Na and K channels. Secondly, Ca entry is elicited through the activation of voltage-dependent Ca channels. Thirdly, intrasynaptosomal events occurring distal to Ca entry orchestrate the exocytosis, retrieval and recruitment of synaptic vesicles. Modulation of release is therefore potentially possible by action at any one or more of these loci. In this paper, I will examine the evidence for the role of protein phosphorylation in the modulation of glutamate release. I will firstly consider the role of protein kinase C (PKC) in regulating release by affecting plasma membrane depolarization. I will

Abbreviations used: 4AP, 4-aminopyridine; Ca/CAM KII, Ca/calmodulin-dependent kinase II; MARCKS, myristoylated, alanine-rich C-kinase substrate; PDBU, 4β-phorbol dibutyrate; PKC, protein kinase C; SSV, small electrolucent synaptic vesicle; TTX, tetrodotoxin.

*Present address: Department of Pharmacology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.