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Role of the Growth-Associated Protein B-50/GAP-43 in Neuronal Plasticity

W. H. Gispen, H. B. Nielander, P. N. E. De Graan, A. B. Oestreicher, L. H. Schrama, and P. Schotman*

Rudolf Magnus Institute, Institute of Molecular Biology and Medical Biotechnology, and Laboratory for Physiological Chemistry, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Contents

*Author to whom all correspondence and reprint requests should be addressed.

Abstract

The neuronal phosphoprotein B-50/GAP-43 has been implicated in neuritogenesis during developmental stages of the nervous system and in regenerative processes and neuronal plasticity in the adult. The protein appears to be a member of a family of acidic substrates of protein kinase C (PKC) that bind calmodulin at low calcium concentrations. Two of these substrates, B-50 and neurogranin, share the primary sequence coding for the phospho- and calmodulin-binding sites and might exert similar functions in axonal and dendritic processes, respectively. In the adult brain, B-50 is exclusively located at the presynaptic membrane. During neuritogenesis in cell culture, the protein is translocated to the growth cones, i.e., into the filopodia. In view of many positive correlations between B-50 expression and neurite outgrowth and the specific localization of B-50, a role in growth cone function has been proposed. Its phosphorylation state may regulate the local intracellular free calmodulin and calcium concentrations or vice versa. Both views link the B-50 protein to processes of signal transduction and transmitter release.

Index Entries: B-50; GAP-43; neuromodulin; growth-associated proteins; cDNA cloning; protein kinase C; calmodulin binding; immune localization; pheochromocytoma (PC12) cells; NGF induction; growth cones; transmitter release.

Introduction

Expression

The protein B-50 was originally described as a phosphoprotein in adult rat brain (Zwiers et al., 1976), where it is located at the presynaptic membrane (Gispen et al., 1985a). The identical protein GAP-43 was described as a prominent member of the family of growth-associated proteins (GAPs) in the nervous system. These represent a relatively small subset of proteins synthesized at strikingly higher levels during neurite outgrowth (Skene and Willard, 1981). It has been proposed that the induction of members of this family of GAPs is a prerequisite for axonal growth (Levine et al., 1981). The best characterized member of the family is GAP-43/B-50, in goldfish named GAP-48 (Benowitz and Lewis, 1983). It is consistently expressed during developmental and regenerative axonal growth and is delivered by fast axonal transport to the extending neurite, especially to the growth cones (Skene and Willard, 1981). The presumed mediating role of B-50 in neurite growth is documented by a large number of studies in different neuronal systems during the last decade (Benowitz and Routtenberg, 1987; Allsopp and Moss, 1989; Benowitz et al., 1989,1990; De la Monte et al., 1989; Erzurumlu et al., 1989,1990; Gorgels et al., 1989; Hoffman, 1989; O'Hara et al., 1989; Skene, 1989; Tetzlaff et al., 1989; Van der Neut et al., 1990; Van der Zee et al., 1989; Verhaagen et al., 1989,1990; Baizer et al., 1990; Biffo et al., 1990; DiFiglia et al., 1990; Goslin and Banker, 1990; McIntosh et al., 1990; Moya et al., 1990; Sheu et al., 1990; Woolf et al., 1990; Yamamoto and Kondo, 1990; Yankner et al., 1990).

Neuron Specificity?

Although B-50 is usually referred to as a neuron-specific protein, a number of recent reports show that under certain circumstances, B-50 is present in other cell types of the nervous system (Vitkovic et al., 1988; Tetzlaff et al., 1989; Da Cunha and Vitkovic, 1990; Deloulme et al., 1990; Hammang et al., 1990). The general opinion is that B-50 is restricted to the nervous system and is expressed in most if not all neuronal cells at some stage. During axonal growth or regeneration, this expression is enhanced. In mature undamaged neurons, expression is mostly low, whereas neurons that are believed to be involved in ongoing synaptic remodeling, e.g., in human associative

brain areas (Neve et al., 1987,1988; Ng et al., 1988; Benowitz et al., 1989) and rat hippocampal and olfactory areas (Oestreicher et al., 1986; Oestreicher and Gispen, 1986; Benowitz et al., 1988; De la Monte et al., 1989; Verhaagen et al., 1989) continue to express high levels of B-50.

GAP-43, pp46, F1, P-57, and Neuromodulin

The protein B-50/GAP-43 is also known under several other names, reflecting independent characterization by various approaches: pp46, a major growth cone protein from fetal rat brain (Katz et al., 1985); F1, a rat hippocampal protein implicated in a model of information storage known as long-term potentiation (LTP; Lovinger et al., 1986); or P-57, now called neuromodulin, a calmodulin (CaM)-binding protein from bovine brain (Andreasen et al., 1983). Although the vast amount of data obtained from different fields of neuroscience has greatly expanded knowledge of the protein and its characteristics, the physiological role of B-50 remains to be solved.

Primary Structure of B-50

Anomalous Behavior in SDS-PAGE

Originally characterized as an acidic protein (pI 4.3–4.7), B-50 showed anomalous behavior in SDS-PAGE; the apparent mol wt increases with lowered percentage acrylamide (Schrama et al., 1987; Benowitz et al., 1987). In 11% gels, the rat protein (23.6 kDa) has an apparent mol wt of 48,000. The unusual behavior of B-50 in SDS-PAGE has been used to identify the protein in different neuronal systems (Benowitz and Routtenberg, 1987). It has, however, delayed the recognition that proteins studied under different names by several laboratories were in fact identical. The most notable example is the bovine protein P-57, or neuromodulin, discovered by its CaM-binding properties (Andreasen et al., 1983); its identity with B-50 was only recently recognized (Cimler et al., 1987).

Primary Structure

The primary structure of B-50 has been determined in a growing number of species. In bovine, this was achieved by direct sequencing (Wakim et al., 1987) and in rat (Basi et al., 1987; Karns et al., 1987; Nielander et al., 1987; Rosenthal et al., 1987; Changelian et al., 1990), human (Ng et al., 1988; Kosik et al., 1988), mouse (Cimler et al., 1987), goldfish (Labate and Skene, 1989), and chicken (Baizer et al., 1990) by molecular cloning. So far, the cDNAs reported for one species are identical within the coding region.

The B-50 open reading frame contains two potential initiation codons. In all species studied, the first codon conforms better to the rules formulated by Kozak (1989) for eukaryotic translational start sites than the second (at position 5). In addition, evidence obtained in vitro (Nielander et al., 1990) and in vivo (Basi et al., 1987) suggests that in rat brain, translation starts at the first of the two methionines. Thus, it seems unlikely that the purified B-50 forms that lack the first four residues (Basi et al., 1987; Rosenthal et al., 1987; McMaster et al., 1988; Baudier et al., 1989) represent primary translation products.

It turned out that B-50 is an extremely hydrophilic protein of 226–247 amino acids, dependent on the species. There are no potential membrane-spanning domains in the molecule; a small amino-terminal region forms the only hydrophobic part of B-50. The primary sequence is well-conserved between species; it shows an abundance of charged, mainly acidic residues, a high percentage of alanine and proline, and an almost complete absence of residues with large nonpolar or aromatic side chains.

Secondary Structure

Circular dichroism studies of the bovine protein have indicated that the secondary structure of the protein in the presence of 0.05% Lubrol PX is mainly random coil, with some β-sheet and almost no α-helix (Masure et al., 1986). Based on sucrose density gradient sedimentation and fluorescence polarization measurements, an

axial ratio of approx 16:1 was reported (Masure et al., 1986). These properties are in accordance with the primary structure; for instance, the relative abundance of proline residues would disrupt potential α-helices. The high density of charged residues, the hydrophylicity, and the lack of large, nonpolar side groups promoting internal folding predicts a highly extended structure (Labate and Skene, 1989). Proton nuclear magnetic resonance (NMR) studies gave no indications of ordered secondary structure in B-50 (Coggins et al., 1989).

Protein B-50 shows anomalous behavior in SDS-PAGE. Comparison with proteins showing a simuar anomality (Kleinschmidt et al., 1986; Takano et al., 1988; Graff et al., 1989a) reveals as a common feature the highly charged character, which could reduce SDS binding to the protein. Also, B-50 has been found to bind SDS poorly (Benowitz et al., 1987). The finding that different B-50 peptides show a similar anomality in SDS-PAGE (Nielander et al., 1991) is in accordance with this explanation, since charged residues are spread over the whole molecule.

Homology to Other Proteins

Recently, a 17-kDa neuron-specific protein designated as neurogranin, p17, or RC3 has been characterized as containing some strikingly conserved amino acid sequences with respect to B-50 (residues 1–4 and 34–52) (Represa et al., 1990; Watson et al., 1990; Baudier et al., 1991; Coggins et al., 1991). This might suggest an evolutionary link between neurogranin and B-50, particularly because both are neuron-specific PKC substrates expressed in dendritic and neurite extensions, respectively.

A similarity in amino acid composition but not so much in sequence is found with the particle-bound cytosolic protein CAP-23. This protein was recently discovered by virtue of the similarity of its physical properties (anomalous SDS-PAGE behavior) and subcellular distribution (membrane cytoskeleton-associated) to that of B-50 (Widmer and Caroni, 1990).

Purified Protein and Primary Translation Product

Isolation Procedures

Although it is mainly membrane-bound, B-50 is also found in soluble fractions during purification (Cimler et al., 1985; Skene and Virág, 1989; Changelian et al., 1990; Moss et al., 1990; Costello et al., 1991). Several purification procedures for B-50 make use of the membrane association of the protein, starting with a synaptosomal membrane preparation. The protein is easily extracted from the membrane fraction at high pH (Oestreicher et al., 1983) but not with high salt or ethylenediaminetetraacetic acid (EDTA; Skene and Willard, 1981), agents that usually remove nonintegral membrane proteins, although a combination of salt and detergent can release B-50 to some extent (Rodnight, 1982; Zwiers et al., 1979,1980). Moss et al. (1990) and Meiri and Gordon-Weeks (1990) reported that a considerable part of B-50 from chick and rat brain, respectively, is contained in membrane skeleton preparations, insoluble even at very high concentrations of nonionic detergents.

For further purification of B-50, use has been made of several of its remarkable properties: its extreme acidity for purification from isoelectric focusing (IEF) gels, its resistance to heat and alkali (probably owing to its hydrophylicity), its solubility in 2.5% perchloric acid (a property shared with several other PKC substrates; Baudier et al., 1989), and the specific binding of CaM at low Ca^{2+}, for purification with CaM-Sepharose columns (Andreasen et al., 1983).

Posttranslational Modifications

To date, the only known *in situ* posttranslational modifications of B-50 concern the addition of fatty acid chains to Cys residues (Skene and Virág, 1989), the phosphorylation by PKC (Aloyo et al., 1983) of Ser residues (Chan et al., 1986; Alexander et al., 1987). Posttranslational modifications seem to have little effect on electrophoretic behavior of purified B-50 in SDS-PAGE: The pri-

mary translation product of B-50, obtained by cell-free translation (Nielander et al., 197, 1990), and B-50 obtained by means of an *Escherichia coli* expression system (Au et al., 1989) comigrate in SDS-PAGE with the purified protein. Recently, however, a separation of phospho- and dephospho-B-50 by one-dimensional SDS-PAGE has been reported (Meiri et al., 1991).

Microheterogeneity

Isoelectric focusing in a narrow pH range reveals 3–4 spots for B-50 (Zwiers et al., 1985; Oestreicher et al., 1989; Skene and Virág, 1989; Nelson et al., 1989; Deloulme et al., 1990). A remarkable heterogeneity in purified protein preparations is also encountered in other chromatographic separations (McMaster et al., 1988; Baudier et al., 1989; Schotman et al., 1989). This points to co- or posttranslational modifications, although some of the heterogeneity might be explained by interactions of the highly charged B-50 with chromatographic matrices (Schotman et al., 1989) or by the amphiphatic character of the protein showing an ion dependency for its division into aqueous and detergent phases (Basi et al., 1987; Dosemeci and Rodnight, 1987; Perrone-Bizzozero et al., 1988; Schotman et al., 1989; Skene and Virág, 1989).

The very large isoforms found on gel filtration might be caused by multimer formation (Chan et al., 1986; Masure et al., 1986; Benowitz et al., 1987; Schotman et al., 1989). There are also indications that B-50 is complexed to other proteins in the cell. After detergent extraction from the membrane, during a number of chromatographic purification steps, B-50, PKC, and phosphatidylin-ositol 4-phosphate (PIP) kinase remained in one complex (Zwiers et al., 1980; Aloyo et al., 1983; Van Dongen et al., 1985). Indeed, B-50 appears to be part of a large hydrophilic complex (Chan et al., 1986; Benowitz et al., 1987).

With respect to the observed heterogeneity of phosphorylated B-50, it has been argued (Schotman et al., 1989) that this cannot be fully explained by different phosphorylation states mediated by PKC and phosphatases, in conjunction with fatty acylation of Cys3 and -4, because only one phosphorylation site for PKC has been found. This suggests the existence of additional modifications or interactions of B-50. No evidence could be obtained for any *N*-linked glycosylation of B-50 (Skene and Willard, 1981; Zwiers et al., 1985; Masure et al., 1986; Benowitz et al., 1987). Autophosphoryl-ation of B-50 is unlikely since the primary structure of B-50 contains no potential ATP-binding sites. Obviously, membrane-associated and soluble B-50 might represent different isoforms of the protein. If not artifacts of the isolation procedure, B-50 isoforms lacking the first four residues (the sole hydrophobic part of the molecule) are good candidates for a soluble protein fraction (Skene and Virág, 1989). Products of B-50, resulting from specific proteolysis by brain enzymes, are B-60 (B-50 41-226) and a phosphorylation-inhibiting peptide (B-50 1-40; Zwiers et al., 1982; Zwiers and Coggins, 1990), and a 40-kDa protein (B-50 1-203; Oestreicher et al., 1989).

In metabolic labeling studies, the onset of charge heterogeneity was observed only after several hours, suggesting that the heterogeneity is caused by a modification that does not take place in the cell body where B-50 is translated (Skene and Virág, 1989). Interestingly, B-50 from cultured oligodendrocytes migrates as a single spot in IEF gels (Deloulme et al., 1990). This raises the question of whether there is a neuron-specific mechanism behind the heterogeneity observed in neuronal cells.

Domains in B-50

Current knowledge of the protein structure and organization of the B-50 gene enables the assignment of certain functions to specific domains of the molecule. The main functional domains are contained by the amino terminal part, residues 1–56; those include sites for membrane association, phosphorylation by PKC, and CaM binding (Fig. 1).

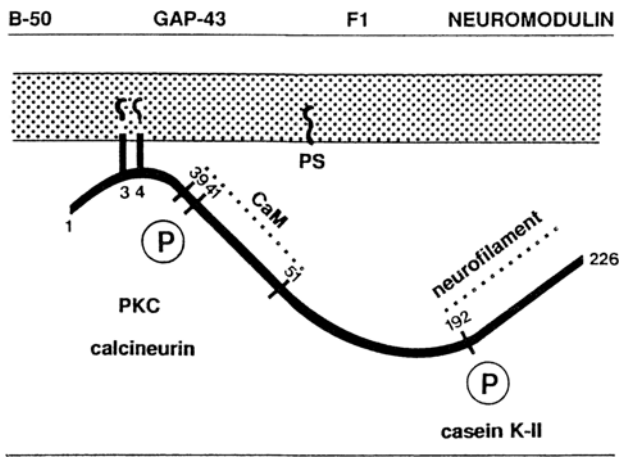

Fig. 1. Functional domains in rat B-50 alias GAP-43 alias F1 alias neuromodulin. PKC, protein kinase C; CaM, calmodulin; P, phosphate; and PS, phosphatidylserine, indicate proposed sequence for CaM or neurofilament interaction. Numbers refer to rat B-50 aminoacid sequence.

Gene Structure

The protein is encoded by three exons in the rat (Grabczyk et al., 1990) and human gene (Schrama et al., 1990). Often, exons correspond to functional domains in a protein (Gilbert, 1986). The first exon encodes the amino-terminal 10 residues of B-50, responsible for the membrane association of the protein (Zuber et al., 1989a). The major part of B-50, including CaM-binding and phosphorylation sites, originates from a 568 or 595bp exon (in rat and human, respectively); the last 27 or 28 amino acids are encoded by a third exon.

Based on comparison of primary sequences of a number of mammalian species and goldfish, a division was made by Labate and Skene (1989) in a highly conserved *N*-terminal sequence of 57 residues and a large C-terminal part with a marked conservation in amino acid composition. Phylogenetic comparison has been used to locate sequences of possible interest, especially in the C-terminal part (Labate and Skene, 1989; Moss et al., 1990).

The distribution of prolines and cysteines in a protein is of special importance. Disulphide bridges between cysteines are main determinants of tertiary structure, and clusters of prolines, for instance, can delineate functional domains. The *N*-terminal 59 residues of B-50 contain only one proline at position 27, whereas the remainder of B-50 is proline-rich, with an average distance of 10 residues and one adjacent pair of prolines at positions 184 and 185. (Residue numbering is based on the rat amino acid sequence; in other species, the distribution is very similar.)

Membrane Association

The small amino-terminal hydrophobic part of B-50 (Met-Leu-Cys-Cys-Met) seems insufficient to explain the association of B-50 with the membrane. Many eukaryotic proteins contain covalently attached lipid, e.g., fatty acid, bound by amide, thioester, or O-acyl bounds (Schmidt, 1983; Low, 1987; Magee and Hanley, 1988). A remarkable feature of the B-50 amino-terminus is the presence of two adjacent cysteines (residues 3 and 4), the sole ones in the protein. Skene and Virág (1989) presented evidence for palmitoylation of B-50, presumably through a thioester bond at Cys3 and/or -4. Soon after synthesis in the cytosol, the protein becomes membrane-associated (Skene and Virág, 1989). The group of Benowitz could not detect acylation of B-50 in cultured embryonic neurons (Perrone-Bizzozero et al., 1989). The data from Skene and Virág (1989) suggest that acylation of B-50 is a dynamic process; in isolated growth cones, labeled palmitic acid was incorporated into B-50. For other proteins known to contain covalently bound fatty acids, often the attached lipid does not seem to be responsible for direct anchoring of the protein to the membrane (Schmidt, 1983; Low, 1987). The punctuate staining for B-50 observed in growing axons (Meiri et al., 1988) seems inconsistent with a direct interaction of B-50 with the lipid bilayer. Like B-50, the neural-specific protein SCG10 is tightly membrane-associated but lacks a membrane-spanning hydrophobic segment; it also contains two cysteines at positions 22 and 24. The intracellular localization of SCG10 resembles that of B-50, including the punctuate staining in growth cones (Stein et al., 1988).

The B-50 amino terminus was found to be blocked for direct amino acid sequencing (Rosenthal et al., 1987; McMaster et al., 1988; P. Schotman and L. H. Schrama, unpublished results), even after alkaline extraction, which largely uncouples acyl chains linked by a thioester bond. This indicates that still other posttranslational modifications may appear at the *N*-terminus. Proton NMR studies indicated the possible attachment of a polyisoprenoid to B-50 (Coggins et al., 1989; Coggins and Zwiers, 1991).

Whatever the exact nature of the modifications at the *N*-terminus, there is convincing evidence that the first 10 amino acids of B-50 contain all the determinants for membrane association. Zuber et al. (1989a) constructed recombinant proteins of the first 10 residues of B-50 with chloramphenicol acetyl transferase, a cytosolic protein. This appeared sufficient to direct the fusion protein to the membrane in transfected "neuronal" (PC12) and nonneuronal (COS) cell lines. Moreover, the fusion protein had the same distribution in PC12 (pheochromocytoma) cells as normal B-50. Similar results in PC12 cells with a fusion of B-50 1-10 to another cytosolic protein, β-galactosidase, were reported by Neve et al. (1990). Mutation of Cys3 and/or -4 in B-50 caused the protein to remain mainly cytosolic (Zuber et al., 1989a), confirming the importance of both residues for membrane targeting.

Interaction With G_0 Protein

Trimeric GTP-binding (G) proteins transduce information from transmembrane receptors to intracellular systems (Gilman, 1987). The most abundant G protein in brain is G_0, a major growth cone protein. Its exact role is not clear, but it has been implicated in regulation of Ca^{2+} channels and phospholipase C activity (Banno et al., 1987; Brown and Birnbaumer, 1990).

Recently, it was shown that B-50 can regulate the binding of GTP-γ-S to G_0 (Strittmatter et al., 1990). The first 24 residues of B-50 stimulated GTP-γ-S binding to G_0 to the same level as the whole protein (although much higher concentrations of the peptide were needed), whereas the first 10 amino acids did not stimulate this binding but competed with B-50 for binding to G_0 Apparently, both exon 1 (residues 1–10) and exon 2 encode information important for interaction with G_0. Sequence comparison of B-50 with domains of G-linked transmembrane receptors proposed to be involved in G_0 activation reveals a homologous sequence with the consensus amino acid sequence hydrophobic-Leu-Cys-Cys-X-basic-basic (residues 1–7 in B-50; Strittmatter et al., 1990). In the three cases reviewed by Strittmatter et al. (1990), Cys residues in the transmembrane receptor proteins were subject to fatty acid acylation. Since the experiments of Strittmatter et al. were performed with alkali-extracted, presumably, nonpalmitoylated B-50, fatty acid acylation of B-50 may not be essential to the interaction of B-50 with G_0.

The nature of the proposed interaction and its physiological relevance remain to be established. Strittmatter et al. did not succeed in isolation of a G_0/B-50 complex, and modulation of GTPase activity by B-50, the physiological parameter for G_0 function, has to our knowledge not been shown yet.

Phosphorylation by PKC

In general, PKC plays an important role in cellular responses to extracellular stimuli (Miller, 1986; Nishizuka, 1988,1989). In the nervous system, B-50 is a major substrate of PKC. Initially, it was found that in vitro phosphorylation of B-50 was inhibited by adrenocorticotropic hormone ACTH1-24 (Zwiers et al., 1976, 1978). Subsequent studies showed a correlation of the phosphorylation state of B-50 with the activity of a PIP kinase (Van Dongen et al., 1985; Gispen et al., 1985b, 1986; Van Hooff et al., 1988), LTP (Lovinger et al., 1986; Benowitz and Routtenberg, 1987; De Graan et al., 1988a), and the modulation of neurotransmitter release (Dekker et al., 1989a,b, 1990a,b; Heemskerk et al., 1990; De Graan et al., 1991).

Evidence from several laboratories suggested that Ser^{41} might be the unique phospho site for PKC (Coggins and Zwiers, 1989; Nielander et al.,

1989; Apel et al., 1990). The fact that mutation of Ser^{41} into Thr^{41}, Ala^{41}, or Gly^{41} prohibited phosphorylation by PKC in vitro seems to prove this (Nielander et al., 1990; Chapman et al., 1991). Proteolytic digestion of *in situ* phosphorylated B-50 resulted into the same phospho-fragments obtained following in vitro phosphorylation by PKC, indicating that Ser^{41} is of physiological significance (Van Hooff et al., 1988; Heemskerk et al., 1989; Oestreicher et al., 1989).

Phosphate is incorporated into the more acidic forms obtained by IEF and preferentially into the most acidic isoform of B-50 (Zwiers et al., 1985; Skene and Virág, 1989; Deloulme et al., 1990). Since there is a single phosphate acceptor site (Ser^{41}), this preference for one of the isoforms suggests that posttranslational modifications of B-50 (*see* Membrane Association, p6) can regulate phosphorylation by PKC (Deloulme et al., 1990). Dephosphorylation of B-50 can be mediated by several phosphatases in vitro (Liu and Storm, 1989; Schrama et al., 1989; Dokas et al., 1990). The physiological relevance of that is as yet unclear.

Phosphorylation of B-50 by protein kinases other than PKC is still under study. Aloyo et al. (1983), Akers and Routtenberg (1985), and Chan et al. (1986) found no significant in vitro phosphorylation of B-50 from adult rat brain with cAMP-dependent kinase and Ca^{2+}/CaM kinase I and II. Nevertheless, Katz et al. (1985) reported phosphorylation by a Ca^{2+}/CaM-dependent kinase in growth cones. More recently, it has been shown that casein kinase II can phosphorylate B-50 in vitro, presumably at Ser^{192} (rat: Pisano et al., 1988; mouse Ser^{193}: Apel et al., 1991). To date, however, PKC is the only kinase documented to play a role in phosphorylation of B-50 *in situ*.

It remains to be shown to what extent the population of B-50 proteins is phosphorylated during different stages of neuronal maturation or in different subcellular compartments. Phosphorylation by PKC, as judged from the onset of appearance of the more acidic forms of B-50 on IEF in metabolic labeling studies, does not take place until several hours after synthesis in the cell body (Skene and Virág, 1989). From a study performed with monoclonal antibodies (MAb) recognizing specifically the phospho-form, it was concluded that not only is the phosphorylated form spatially restricted to the distal axon and growing tip but that phosphorylation might occur subsequent to the onset of axonogenesis (Meiri et al., 1991).

CaM Binding

The protein B-50 interacts with CaM in an unusual way. It binds to CaM-Sepharose at low Ca^{2+} conditions and can be released by raising the Ca^{2+} concentration (Andreasen et al., 1983), whereas most other CaM-binding proteins show a Ca^{2+} dependency for CaM binding. The interaction of neuromodulin (B-50) with CaM has been studied in detail by the group of Storm (Liu and Storm, 1990; Estep et al., 1990; Chapman et al., 1991). Also, B-50 crosslinks to a CaM derivative in a 1:1 molar ratio (Andreasen et al., 1983), indicating that it has a single CaM-binding site. In accordance with its unusual CaM-binding characteristics, the structural properties of B-50 distinguish it from CaM-binding regions in other proteins (mainly basic amphiphilic α-helical peptides (O'Neil and Degrado, 1990). Using proteolytic fragments and synthetic peptides of B-50, it was shown that the CaM-binding site encompasses residues 43–51 (Alexander et al., 1988). Interestingly, this domain weakly satisfies the proposed criteria for a "normal" CaM-binding site; it is the only region carrying a net positive charge, and it contains the only aromatic residue in B-50.

The interference of phosphorylation by PKC with CaM binding (Alexander et al., 1987; Graff et al., 1989a) suggests that Ser^{41} forms part of the binding site. Interference by a phosphate group may be caused by steric hindrance, the introduction of extra negative charges (mimicked by mutating Ser^{41} into Asp^{41} (Chapman et al. 1991), an effect on the adjacent Phe residue, or an alteration of secondary structure, leading to a decreased affinity for CaM.

A comparable interaction between CaM binding and PKC-mediated phosphorylation is

observed for the myristoylated alanine-rich C kinase substrate (MARCKS) or 87-kDa protein, a protein of unknown function (Graff et al., 1989b). This protein shares other characteristics with B-50, such as enrichment in growth cones, acidity, and anomalous electrophoretic behavior in SDS-PAGE. Its interaction with CaM, however, is Ca^{2+}-dependent, and MARCKS is not restricted to the nervous system (Albert et al., 1987; Graff et al., 1989b).

The reduced CaM binding reported for B-60 (amino acids 41–226) suggests a possible involvement of sequences *N*-terminal to Ser^{41} in the interaction of B-50 and CaM (Coggins and Zwiers, 1990)—more explicitly residues 38–40, since a synthetic peptide 38-55 bound CaM with an affinity comparable to native B-50 (Alexander et al., 1988).

Two other proteins have been described to bind to CaM-Sepharose with characteristics similar to B-50. The rat adipocyte protein pp170 (McDonald and Lawrence, 1989) and a protein of 16 kDa found in rat brain and SPM that is alkaline-resistant and heat-stable as well (Schrama et al., 1989), recently characterized as p17/neurogranin (Baudier et al., 1991), BICKS (B-50-immunoreactive C-kinase substrate, Coggins et al., 1991), or RC3 (Watson et al., 1990). Both proteins are also subject to phosphorylation by PKC.

Alexander et al. (1987) observed in vitro a reduced affinity of B-50 for CaM with increasing ionic strength. This and the structure of the proposed CaM-binding domain suggest that the B-50–CaM interaction is primarily ionic in nature (Alexander et al., 1988). The dependency on low calcium concentrations and the effects of ionic strength in vitro have raised questions on the physiological significance of the CaM–B-50 interaction (Skene 1989; Coggins and Zwiers, 1991). However, in native synaptosomal and growth cone membranes, complex formation between CaM and endogenous B-50 was shown with the crosslinker disuccinimidyl suberate (DSS) under physiological Ca^{2+} concentrations (De Graan et al., 1990). Moreover, the Ca^{2+} sensitivity of complex formation was not decreased by raising the ionic strength (P. N. E. De Graan, unpublished results) as described for the in vitro system (Alexander et al., 1987). Recently, Houbre et al. (1991) demonstrated that the conserved amino acid sequence in B-50 and neurogranin that corresponds to the PKC phosphorylation site and CaM-binding domain is essential for the binding to phosphatidylserine, which mediates the phosphorylation by PKC.

Interaction with Cytoskeletal Elements

The region C-terminal to residue 57 shows a sharp divergence in primary sequence between rat and fish (Labate and Skene, 1989). Several short regions of apparent sequence conservation between fish and mammalian species, potentially involved in interactions with other proteins, have been noted in this part of B-50 (motifs A–F; Labate and Skene, 1989). In motif B, repeated twice in the rat and human, the sequence (SEEK) is found noted for its similarity to the PKC phosphorylation site of the α-subunit of the nicotinic acetylcholine receptor (Nielander et al., 1987). In rat B-50, however, there seems to be no significant phosphorylation by PKC of these sites (Nielander et al., 1990).

Sequence motif C (ETXESSQ) contains the postulated site for phosphorylation by casein kinase II (rat residue 192, mouse residue 193; Pisano et al., 1988; Apel et al., 1991). Indeed, the region containing motif C and part of motif F shows some homology to a casein kinase consensus sequence in the c-*myc* proto-oncogene product *myc* (Coggins and Zwiers, 1991). Ng et al. (1988) noted several internally homologous direct repeat regions in the human B-50 cDNA, forming a contiguous domain near the COOH-terminal end. In exon 3, coding for the 28 amino acids at the C-terminus, some structural similarity was found for goldfish B-50 and a mouse neurofilament (NF-L).

This homology might be responsible for the observed immunological crossreactivity between some B-50 antisera and NF-L proteins (G. J. A.

Ramakers, personal communication). The homologous sequence is located in a part of NF-L thought to be involved in interaction with other cytoskeletal elements. This structural similarity has led to the proposition that B-50 might interact with skeletal elements through its C-terminal domain (Labate and Skene, 1989).

Although there is little sequence homology, the amino acid composition of the large C-terminal part (residues 57–226 in rat) is strongly conserved, and such predicted physical characteristics as hydrophylicity and charge are very similar between species (Labate and Skene, 1989). The functionally important feature, which apparently can be accommodated by quite-relaxed sequence requirements, might be the extended, negatively charged rod-like structure (Labate and Skene, 1989). Recently, B-50 has been proposed to extend from the cytoplasmic side of the membrane inward (Gispen et al., 1985a; Meiri et al., 1988). With the amino terminus involved in membrane association and an extended structure, this would bring the carboxyl-terminus away from the membrane in the cytosolic space. The recent characterization of B-50 as a component of the neuronal membrane skeleton in chicken and rat (Allsopp and Moss, 1989; Moss et al., 1990; Meiri and Gordon-Weeks, 1990) is consistent with such a view.

Functional Aspects of B-50

Posttranslationally, B-50 undergoes several modifications. Some of these, i.e., phosphorylation and palmitoylation, are reversible, involving fast-working enzymes. Specific proteolytic cleavage, e.g., C-terminally to residues 4 and 41, might provide alternative, irreversible ways to modify the protein. The general picture arises of a series of B-50 isoforms, with various types of modifications, perhaps related to differential localization within the cell. These different isoforms are capable of interacting with a number of other functional proteins, some of which are thought to be part of a multifunctional B-50-containing protein complex at the inner surface of the plasma membrane.

Role in Neuritogenesis

A multitude of functions has been proposed for B-50 in neuronal growth, receptor-mediated processes, and transmitter release. So far, a unifying concept is missing. The best documented role is in neuronal growth (for refs., *see* Introduction), albeit that a causal relationship between growth and B-50 expression is still under debate (Gordon-Weeks, 1989; Megerian and Klein, 1990; Yankner et al., 1990; Baetge and Hammang, 1991; Costello et al., 1991). Important arguments in favor of such causal relationships are the following:

1. Transfection of COS cells with an expression vector containing this B-50 gene leads to formation of growth cone-like filopodia (Zuber et al., 1989b);
2. Transfected PC12 cells that overexpress B-50 display enhanced responsiveness to nerve growth factor (NGF) and regeneration of damaged neurite-like extensions (Yankner et al., 1990); and
3. In rat, PC12 cells antisense oligomers to B-50 blocked the NGF-induced increase in B-50 expression and at the same time, the enhanced neurite outgrowth (Schotman et al., 1990).

Future research should shed light on the precise role of B-50 in neuritogenesis.

Role in NGF-Induced Neuronal Differentiation of PC12 Cells

Pheochromocytoma cells have been intensively studied as a model for the mechanism of neuronal differentiation. When rat PC12 cells are cultured in the presence of NGF, the cells stop proliferating and differentiate to a phenotype resembling cholinergic sympathetic neurons, elaborating neuritic extensions (Greene and Shooter, 1980). Also, B-50 has been identified in undifferentiated and NGF-treated PC12 cells (Van Hooff et al., 1989b). Treatment of PC12 cells

with NGF for 2 d results in a 2.5-fold increase in the B-50 level as measured by radioimmunoassay (RIA). Immunofluorescence microscopy shows that B-50 is detectable in fixed permeabilized PC12 cells, as intensified membrane-associated staining in the neurites, especially in the growth cones, of the NGF-differentiated cells and as diffuse staining in the cell bodies of the controls and the NGF-differentiated cells. In contrast, when living, nonpermeabilized PC12 cells are incubated with B-50 antibodies, then fixed and probed with a fluorescein-conjugated secondary antibody, no immunofluorescence is detectable, suggesting that B-50 is inside the cells.

Translocation of B-50 with NGF-Induced Neuritogenesis

With the aim to learn more about the putative growth-associated function of B-50, we have compared its ultrastructural localization in control proliferating PC12 cells, with its distribution in PC12 cells induced to differentiate by various agents (50 ng/mL NGF, 1 m*M* dibutyryl cAMP and substratum). The studies were performed on cells fixed in 2% paraformaldehyde/0.1% acrolein in PBS, embedded in gelatin, cryoprotected, and sectioned on an ultracryomicrotome at –90°C. Grids holding 70–100 nm thick sections were incubated with B-50 antibodies and the antigen-antibody complexes were detected by protein-A gold (approx 10 nm) conjugates (Van Hooff et al., 1989a).

To our surprise, we found that in the cryosections of untreated control PC12 cells, the B-50 immunoreactivity (BIR) is mainly associated with intracellular membrane-rich and vesicular structures, including organelles of the lysosome family and Golgi apparatus. The plasma membrane is virtually devoid of label. In contrast, after 48 h of treatment with NGF or cAMP, BIR is most pronounced on the plasma membrane. The highest BIR is observed on plasma membrane surrounding sprouting microvilli, lamellipodia, and filopodia.

In addition, extending neurites and growth cones contain cytosolic BIR, which is partially associated with chromaffin granules. In the NGF-differentiated PC12 cells, the cytosolic location of B-50 is similar to untreated cells. Although the induction of neurite formation in PC12 cells by NGF and cAMP is reported to occur by different pathways, B-50 distribution in dibutyryl cAMP-differentiated cells closely resembles that observed in the NGF-treated cells. Thus, in PC12 cells, various differentiating agents induce a change in the ultrastructural localization of B-50, facilitating association of the B-50 protein with the plasma membrane (Van Hooff et al., 1989b).

Recently, further ultrastructural studies of the time course of the NGF effect on B-50 distribution indicate that after approx 4 h, the beginning of the shift of B-50 to the plasma membrane can be observed, and after 8 h, the translocation appears to be fully established. When PC12 cells are grown for the same time (48 h) in the presence of epidermal growth factor (EGF, 50 ng/mL), a growth factor inducing proliferation in PC12 cells, the cell number increases, the B-50 levels increase by 50%, but few neuritic protrusions are formed. The ultrastructural distribution of B-50 in the case of EGF is rather similar to that in the untreated control cells (A. B. Oestreicher, unpublished results). This indicates that the translocation of B-50 appears to be linked to initiation and formation of neuritic protrusions. Below we discuss that after translocation of B-50 to the plasma membrane as an initial step in the neuronal differentiation, there follow stages of development in which the neuron undergoes polarization, and B-50 becomes restricted to the axonal domain.

Localization in Growth Cones

The subcellular distribution of B-50 in growth cones was studied by immunogold labeling of B-50 antibodies in ultrathin cryosections of isolated neuronal growth cones (Pfenninger, 1986). Ultrastructure of isolated neuronal growth cones clearly shows a dense matrix of smooth endoplasmic reticulum, round and oval vesicles of various sizes, and small mitochondria. Eighty percent of the B-50 immunoreactivity is located at the plasma membrane, whereas 20% is present

in the cytoplasm of the growth cone, occasionally in the proximity of vesicles (Van Lookeren Campagne et al., 1990). In other electron microscopic immunocytochemical studies on hippocampus (Van Lookeren Campagne et al., 1990) and pyramidal tract axons (Gorgels et al., 1989) during postnatal development of rat brain, we found that B-50 is predominantly located at the plasma membrane of growth cones *in situ*.

Changes in B-50 Distribution During Neuronal Polarization

During development of the nervous system, neurons go through a developmental stage (polarization) in which neurons form two distinct classes of morphological and functional different processes: the axon and the dendrite. In studies of neuronal cell cultures dissociated from embryonic brain (Meiri et al., 1986,1988; Goslin et al., 1988; Burry et al., 1991; Ramakers et al., 1991), it has been observed that during the first days in vitro, the neurons produce short neurites with growth cones, both detectable by dense B-50 immunostaining. As the neurons develop, mature, and make connections, B-50 immunostaining is reduced in the processes and in some cases disappears completely.

For the development of embryonic hippocampal neurons in cell culture, Dotti et al. (1988) have documented the sequence of changes in neurite outgrowth and morphology, leading to polarization and maturation of the neurons. Goslin et al. (1988,1990; Goslin and Banker, 1990) showed by immunofluorescence that after morphological polarization of the pyramidal neurons, B-50 becomes selectively located in the axonal growth cone. In addition, B-50 is demonstrated to be absent from dendrites and their growth cones, identified by microtubule-associated protein-2 (MAP-2), a cytoskeletal marker for dendrites (Dotti et al., 1987).

Our immuno-electron microscopic studies of hippocampal neurons in cell culture at consecutive developmental stages show that B-50 immunoreactivity becomes restricted to the axonal domain, being located at the plasma membrane of varicosities, some parts of the shaft and terminals. A condition for this restriction appears to be that the neurons make contacts and form synapses (Van Lookeren et al., 1991b; Van der Neut et al., 1990).

Evidence for this suggestion is the finding that polarized neurons with a long, thin MAP-2 negative axon grown solitary for more than 1 wk in culture display B-50 immunoreactivity at the plasma membrane of the MAP-2 positive dendrites and growth cones. Since the anti-B-50 antibodies used do not crossreact with neurogranin (A. B. Oestreicher, unpublished results), the immunoreactivity represents only B-50 protein. Hippocampal neurons cultured for 2–3 d in vitro initiate polarization by rapid outgrowth of one longer process in comparison to the other shorter, slower growing neurites. At this stage of development, we have detected B-50 immunoreactivity in all processes by immunofluorscence and ultrastructurally to be homogenously distributed at the plasma membrane of growth cones, the axon, the dendrites, and the cell body. These results suggest that B-50 is not involved in functions exclusively specific for the growth cone but rather is functionally important for the neuronal membrane in general *(see below)*.

In connection with the suggestion that B-50 is not specific for the growth cone, it is relevant to note that in the adult animal, dense B-50 immunolabeling is also detected at the plasma membrane of axon terminals and at that of unmyelinated thin axons in various regions (hippocampus, periaquaductal gray, olfactory system) of the central nervous system (CNS) (Fig. 2; Van Lookeren Campagne et al., 1990,1991a,b) and only little B-50 immunoreactivity in myelinated axons (Gorgels et al., 1989). Of interest is that in the mature CNS, B-50 immunoreactivity has been rarely detected in dendrites, although DiFiglia et al. (1990) have detected B-50 immunuoreactivity not just in unmyelinated fibers and axon terminals but also in dendritic spines of the neuropil of rat neostriatum.

A recent study by Meiri et al. (1991) using two MAb to B-50 with distinct different specificity, one recognizing selectively the PKC-phosphory-

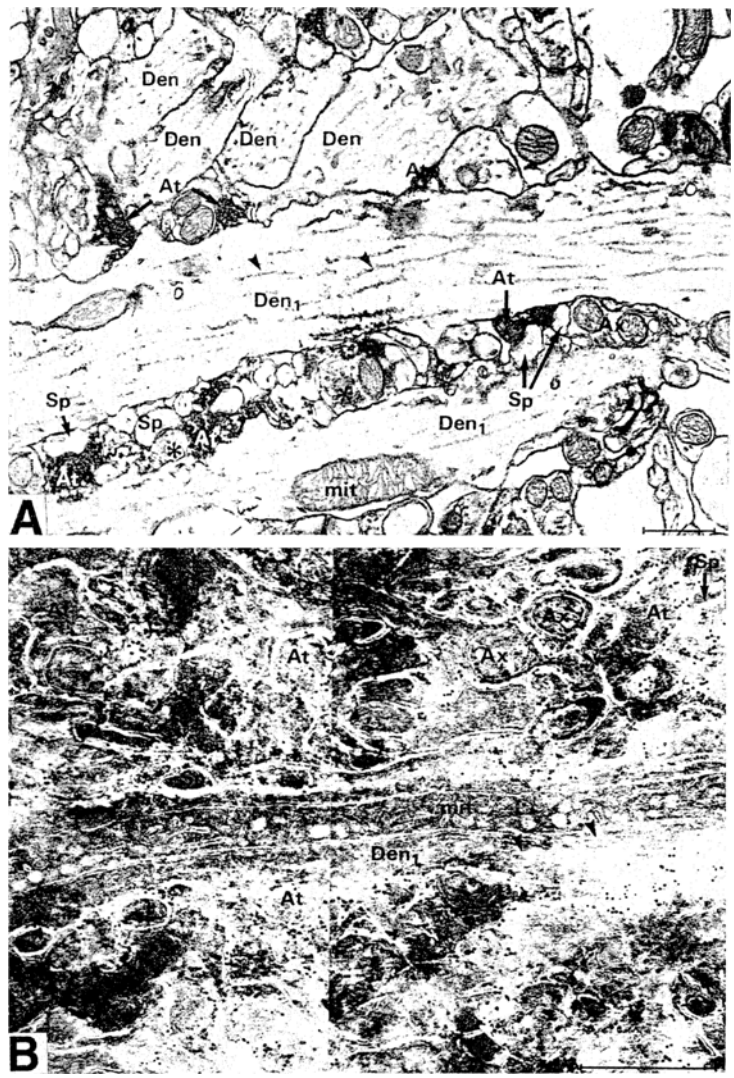

Fig. 2. Electron micrograph of apical dendrites, proximal to the pyramidal cells of the CA1 stratum radiatum in a P90 rat. The sections were immunostained for B-50 by preembedding immunoperoxidase labeling (**A**) or by postembedding immunogold labeling (**B**). Note the absence of immunoreactivity in the longitudinally-sectioned apical dendrites (Den_1), clearly slowing microtubules (arrow heads **A**, **B**). In the preembedding immunostained section, axon terminals (At) are most prominently stained, whereas in the postembedding immunogold (10 nm) labeled section, plasma membranes of all axon shafts (Ax) are labeled for B-50, in addition to those of the axon terminals. Sp, dendritic spine; mit, mitochondrion. Asterisks in **A** show unlabeled axon terminals. Scale bar: 0.5 μm.

lated isoform of B-50 and the other reacting with both the PKC-phosphorylated and nonphosphorylated B-50, shows that during development of nervous tissue, the ratio of these forms is changed, and PKC-phosphorylated B-50 is restricted temporally and spatially to the distal part of the outgrowing axons. Taken with recent ultrastructural data, one of the implications is that the functional state of B-50 is determined locally by the steady-state level of calcium and the dynamic balance between the activities of PKC, protein phosphatases, and other enzymes.

Role of B-50 Phosphorylation in Signal Transduction in the Growth Cone

Nerve growth cone membranes isolated from fetal and neonatal rat brain are enriched in B-50 (De Graan et al., 1985; Katz et al., 1985). Phosphorylation of B-50 in these growth cone membranes in vitro is inhibited by $ACTH_{1-24}$ and stimulated by phorbol diesters, indicating that PKC is the active protein kinase. In addition, $ACTH_{1-24}$ stimulated the phosphatidylinositol 4,5-bisphosphate (PIP_2) formation in growth cone membranes, suggesting the possibility of an inverse relationship between the degree of B-50 phosphorylation and PIP_2 labeling as shown for adult presynaptic membranes (Van Hooff et al., 1988).

An important question is whether extracellular signals can influence B-50 phosphorylation in intact nerve growth cones. To explore this, intact growth cones were isolated from the brain of 5-d-old rats and studied after prelabeling with [^{32}p]- orthophosphate, using an immunoprecipitation method to selectively monitor the degree of B-50 phosphorylation. Phorbol 12,13-dibutyrate and dioctanoyl glycerol, activators of PKC, stimulate B-50 phosphorylation in the intact growth cones, confirming that PKC is involved in B-50 phosphorylation (Van Hooff et al., 1989a).

Depolarization induced by 30 m*M* K^+ produces a transient rise in B-50 phosphorylation. This response to K^+ depolarization is also demonstrated in adult rat brain preparations, such as the hippocampal slice (Dekker et al., 1989b) and isolated synaptosomes. The effect of K^+ depolarization on B-50 phosphorylation in growth cones can be blocked partially by atropine (0.1–1 m*M*). The suggestion of involvement of muscarinic receptors is supported by the finding that carbachol, a cholinergic receptor agonist, enhances B-50 phosphorylation in a concentration-dependent manner (50% at 1 m*M*). The carbachol (1 m*M*) stimulation can be blocked by atropine (0.1 μ*M*). The carbachol stimulation can be further increased by concurrent K^+ depolarization (Van Hooff et al., 1989a,b). It is not yet clear how the receptor-mediated changes in B-50 phosphorylation in isolated growth cones relate to modulation of GTP-γ-S binding to G_0 (Strittmatter et al., 1990).

B-50 Phosphorylation and LTP

To investigate the role of B-50 phosphorylation in long-term potentiation (LTP), Routtenberg and co-workers (Linden and Routtenberg, 1989) elicited LTP by in vivo tetanization and subsequently measured B-50 phosphorylation using [γ-^{32}P]-ATP. They found that tetanization is associated with an increase in post hoc B-50 phosphorylation and membrane-bound PKC activity. Using [^{32}P]-orthophosphate labeling combined with immunoprecipitation of B-50, we measured B-50 phosphorylation in intact hippocampal slices after short-term phorbol ester treatment (De Graan et al., 1988b). The phorbol ester induced a long-lasting enhancement of synaptic efficacy resembling in many ways tetanus-induced LTP, which was accompanied by a long-lasting increase in B-50 phosphorylation (Fig. 3).

Recently, Gianotti et al. (1991) showed that in hippocampal slices (using our immunoprecipitation procedure), LTP is accompanied by an increase in B-50 phosphorylation. It is not yet known how long the increase in B-50 phosphorylation persists after tetanization. It is not yet clear whether B-50 phosphorylation is involved in the presynaptic mechanism underlying LTP. An intriguing possibility is that a long-term increase in B-50 phosphorylation is related to the prolonged increase in neurotransmitter release that has been reported to occur after tetanization (Dolphin et al., 1982).

Role of B-50 in Presynaptic Signal Transduction and Neurotransmitter Release

In view of the possible involvement of PKC in transmitter release and of the presynaptic localization of the PKC substrate B-50, we started a series of studies to investigate the relationship between B-50 phosphorylation and transmitter release.

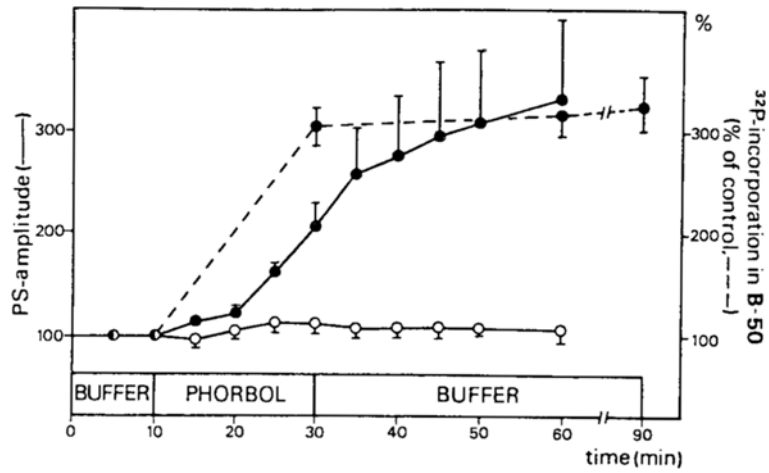

Fig. 3. Long-lasting effects of a 20-min phorbol dibutyrate (PDB) treatment on population spike (PS) amplitude B-50 phosphorylation. After treatment with $10^{-6}M$ PDB (●) or 4α-phorbol didecanoate (4α-PDD)(o), slices were incubated in or perfused with KRB. Field potentials were recorded in the stratum pyramidale of CA1. The average PS amplitude (slid lines, mean ± SEM; and $n = 4$) is expressed as a percentage of the PS during the 10-min control period (prior to the phorbol treatment). B-50 phosphorylation (broken lines, mean ± SEM; and $n = 4$) is expressed as a percentage of the value obtained for untreated control slices at each time point.

Stimulation of transmitter release from rat brain synaptosomes by high K^+, 4-amino-pyridine, or veratridine induces a concomitant phosphorylation of B-50 (Dekker et al., 1990a; Heemskerk et al., 1990). We more closely investigated K^+-induced phosphorylation of B-50. Like K^+-evoked release, K^+-evoked B-50 phosphorylation is dependent on extracellular Ca^{2+} (Dekker et al., 1989a, 1990a). Most likely, both processes are activated by an influx of extracellular Ca^{2+} into the synaptic terminal. K^+-evoked B-50 phosphorylation depends on PKC activity because it is inhibited by polymyxin B, H-7, and staurosporine, three inhibitors of PKC (Dekker et al., 1989a, 1990).

In synaptosomes, K^+ and veratridine-induced phosphorylation of B-50 is transient; it is rapidly induced and returns to basal levels within 5 min (Dekker et al., 1990b). Such a time-dependency profile has also been reported for the amount of transmitter released/unit of time from synaptosomes after depolarization and for the amount of Ca^{2+} entering the synaptosomes for each unit of time (De Graan et al., 1991; Dekker et al., 1991), indicating that Ca^{2+} influx, PKC-mediated B-50 phosphorylation, and transmitter release may be closely related.

The clear correlation that has been found between B-50 phosphorylation and transmitter release did not answer the question of to what extent B-50 phosphorylation is essential to this process. To address this issue, specific B-50 antibodies that inhibit the phosphorylation of B-50 were tested on transmitter release. Insofar as B-50 is localized at the inner leaflet of the synaptic plasma membrane, exposed toward the cytosol (Gispen et al., 1985a; Van Hooff et al., 1989b), the antibodies needed to be introduced into the synaptosomes without disrupting the release machinery. Anti-B-50 antibodies were introduced using a streptolysin-O (SL-O) permeation technique (Dekker et al., 1989a). The effect of K^+ depolarization on noradrenaline (NA) release can be mimicked in SL-O-permeated synaptosomes by increasing the Ca^{2+} concentration in the incubation buffer from 10^{-8} to 10^{-5} (Dekker et al., 1989a). The effect of Ca^{2+} cannot be attributable to an efflux of cytosolic NA, since Ca^{2+} did not stimulate the SL-O-induced efflux of the cytosolic marker protein lactate dehydrogenase (LDH). This confirms that the Ca^{2+}-dependent part of SL-O-induced NA efflux is vesicular NA release.

Next, permeated synaptosomes were incubated with anti-B-50 IgGs concomitantly with the Ca^{2+} trigger, and NA release and B-50 phosphorylation were measured (Fig. 4). Ca^{2+}-induced release of NA was inhibited by the anti-B-50 IgGs in a concentration-dependent way (Dekker et al., 1989a), as was B-50 phosphorylation. Control IgGs and heat-inactivated anti-B-50 IgGs were ineffective. Neither control nor anti-B-50 IgGs affected NA efflux at 10^{-8} and $10^{-7}M$ Ca^{2+}, showing that the antibodies did not influence the Ca^{2+}-independent efflux. Surprisingly, we found that in contrast to Ca^{2+}-induced NA release, phorbol ester-induced release was not inhibited by the anti-B-50 antibodies. Although we do not currently have an explanation for this, it may indicate that phorbol esters and Ca^{2+} have a different effect on transmitter release.

From these studies, we conclude that B-50 plays a crucial role in the molecular mechanism of NA release from rat cerebral cortex synaptosomes. At this moment, it is not clear whether B-50 is involved in the mechanism of release of transmitters other than NA.

Although it has been established that inhibition of B-50 phosphorylation by specific antibodies is accompanied by inhibition of calcium-induced NA release from permeated isolated nerve terminals, this does not imply that only the degree of B-50 phosphorylation is important for NA release. It is also possible that B-50 antibodies block properties of B-50, other than its phosphorylation or in conjunction with its phosphorylation, which are important to transmitter release. In favor of a direct involvement of PKC-mediated phosphorylation of B-50 in transmitter release is the following evidence:

1. Phorbol esters that directly activate PKC and induce B-50 phosphorylation enhance the release of a variety of neurotransmitters, and this enhancement can be antagonized by the kinase inhibitors polymyxin B, staurosporine, and H-7;
2. Both B-50 phosphorylation and neurotransmitter release can be enhanced by high K^+, veratridine, and 4-aminopyridine;

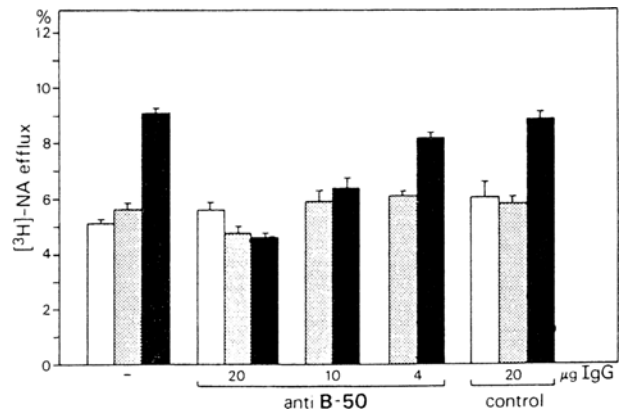

Fig. 4. Effect of anti-B-50 IgG on Ca^{2+}-induced release of NA from SL-O-permeabilized synaptosomes. Synaptosomes were permeabilized with 0.8 IU/mL SL-O and incubated without IgG (–), with different concentrations of anti-B-50 IgG (anti-B-50), or with control IgG (control) (open bars, □ 10^{-8}; dotted bars, □ 10^{-7} M Ca^{2+}; filled bars, ■ 10^{-5} M Ca^{2+}). Neither anti-B-50 nor control IgG (20 µg/assay) affected NA release in nonpermeabilized synaptosomes. Data are expressed as SEM (three independent experiments). Identical results were obtained in a separate experiment using a different batch of and-B-50 IgG. Data were analyzed by one-way analysis of variance (ANOVA) with a supplemental Newman-Keuls test. Asterisk denotes group significantly different ($p < 0.05$) from all groups treated with 10^{-7} or $10^{-8}M$ Ca^{2+}.

3. The protein kinase inhibitor polymyxin B inhibits K^+-induced B-50 phosphorylation and K^+-induced neurotransmitter release; and
4. Anti-B-50 IgGs inhibit B-50 phosphorylation as well as Ca^{2+}-dependent transmitter release.

However, in disagreement with a direct role for PKC-mediated B-50 phosphorylation in neurotransmitter release are the observations that:

1. Phorbol esters enhance B-50 phosphorylation but not transmitter release in polarized tissue;
2. Antibodies to B-50 only partially interfere with transmitter release induced by phorbol esters, which stimulate PKC-mediated phosphorylation of B-50; and
3. The protein kinase inhibitors H-7 and staurosporine, both inhibiting B-50 phosphorylation, do not inhibit Ca^{2+}-dependent transmitter release.

In evaluating these data, it should be emphasized that the precise mechanisms of Ca^{2+} and phorbol ester-induced transmitter release remain to be elucidated. It may very well be that phorbol esters and Ca^{2+} affect neurotransmitter release in a different way, thus giving rise to the differential effects of inhibitors and antibodies on Ca^{2+} and phorbol ester-induced release. Alternatively, as suggested, the present results leave open the possibility that properties of B-50 other than its PKC-mediated phosphorylation are contributing to the transmitter release process and that these are affected by antibodies and inhibitors.

Such properties could involve the CaM-binding capacity of B-50. It has been shown that B-50 binds calmodulin in vitro under low Ca^{2+} conditions and releases CaM when Ca^{2+} is raised (Andreasen et al., 1983; Cimler et al., 1985; De Graan et al., 1990; Liu and Storm, 1990). The phosphorylation site and the CaM-binding site of B-50 are very close to each other (Alexander et al., 1988), and prephosphorylation of B-50 by PKC inhibits its property to bind CaM (Alexander et al., 1987). Data suggest that B-50 acts as a membrane-bound CaM store, releasing CaM after elevation of the Ca^{2+} levels or upon phosphorylation. A depolarization-induced rise of the intracellular concentration of Ca^{2+} in vivo would dissociate CaM and B-50. Once liberated from CaM, B-50 can be phosphorylated by PKC. PKC may be activated by Ca^{2+} entering the synaptic terminal after the depolarization. It is not clear whether such activation should involve the translocation of PKC from the cytosol to the membrane or whether Ca^{2+} directly activates membrane-bound PKC. Alternatively, Ca^{2+} may activate phospholipases generating diacylglycerol which could induce activation of PKC. PKC-mediated phosphorylation could prevent CaM from reassociating with B-50.

Phosphorylation of B-50 is also thought to be a modulatory event in the activity of PIP kinase, the enzyme that converts the membrane lipid PIP to PIP_2 (Gispen et al., 1985b). In fact, it has been shown that an inverse relationship exists between B-50 phosphorylation and PIP_2 labeling (Gispen, 1986). For instance, inhibition of B-50 phosphorylation in synaptosomal plasma membranes by $ACTH_{1\text{-}24}$ or by B-50 antibodies induced a stimulation of PIP_2 production (Oestreicher et al., 1983). Enhancement of B-50 phosphorylation by addition of purified PKC to the synaptosomal plasma membranes resulted in an inhibition of PIP kinase activity (De Graan et al., 1988a).

These data are consistent with the hypothesis that B-50 phosphorylation exerts a negative feedback control on PIP kinase activity. Increased levels of diacylglycerol, formed by receptor-mediated hydrolysis of PIP_2 or phosphatidylcholine, resulting in PKC-mediated B-50 phosphorylation, would reduce replenishment of PIP_2 as a mechanism to reduce further transmembrane signal transduction (Gispen et al., 1985b). It is interesting to note that recently, Houbre et al. (1991) showed that B-50 binds to phosphatidylserine and phosphatidic acid and pointed to a phosphatidylserine affinity site overlapping the phosphorylation site and the CaM-binding domain. Since phosphatidylserine is known as an activator of PIP kinase as well as of PKC, these data support the idea that B-50, PIP kinase, and PKC are part of a multifunctional complex associated with the membrane.

The postulated regulatory effect of B-50 on G_o protein (Strittmatter et al., 1990) could effect PIP_2 hydrolysis in another way, since G_o might be involved in coupling some receptors to phospholipase C (Banno et al., 1987). Furthermore, this proposed interaction of B-50 with G_o forms a link to another important second messenger system of adenylate cyclase and cAMP. It is not yet evident whether phosphorylation of B-50 by PKC is of any relevance to its G_o-modulating effects. Ample evidence exists suggesting the involvement of G proteins in secretory processes (Howell et al., 1987), and it would be very interesting to investigate whether the apparent necessity of B-50 to secretion in the nerve terminal is related to modulation of G_o activity.

An intriguing question remains if there is a general main feature of B-50 responsible for its seemingly diverse roles in developing and mature neurons. A regulatory effect on vesicle fusion pro-

cesses, taking place during axonal growth, synaptic remodeling, and neurotransmitter release seems to be an attractive candidate for such a unifying feature.

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