Molecular Analysis Of The Growth-Associated Protein-43 (GAP-43) Function

by

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CONTENT

FIGURES & TABLES

Abbreviations :

AEDANS-CaM : 5-[[(iodoacetylamino)ethyl]-amino]-1-naphtalenesulfonic acid-labeled calmodulin

BSA : bovine serum albumin

CaM : calmodulin

CRF, corticotropin-releasing factor

DMSO : dimethylsulfoxide

DSP : dithiobis(succinimidyl propionate)

GAP-43 : growth associated protein-43

G-proteins : heterotrimeric GTP-binding proteins

KRH : Krebs-Ringer-Hepes

NGF : nerve growth factor

nt : nucleotide

PVDF : polyvinylidene difluoride

PKC : Ca2+/phospholipids-dependent protein kinase

PIPES : 1,4-piperazinediethanesulfonic acid

RSV : Rous sarcoma virus

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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ABSTRACT

The neuronal growth-associated protein (GAP)-43 is concentrated in the growth cone of elongating axons during development and continues to be expressed in presynaptic terminals of certain neurons in the adult nervous system. GAP-43 is thought to be involved in axon elongation, long term potentiation and neurotransmitter release. As GAP-43, a membrane bound protein, is involved in several second messenger pathways, it may modulate complex cellular properties such as growth cone motility, synaptic plasticity and neurotransmitter release. However, the precise role of GAP-43 in these processes remains to be determined.

A proposed model for the role of GAP-43 in the modulation of secretion is that GAP-43 sequesters calmodulin (CaM) at the inner face of the plasma membrane near its sites of action and releases CaM upon an influx of Ca^{2+}, thus facilitating Ca^{2+}-dependent secretion.

We used the mouse anterior pituitary AtT-20 cell line as a model system to investigate the function of GAP-43 in exocytosis. We established permanent cell lines expressing GAP-43 by transfecting the wild type rat GAP-43 cDNA into AtT-20 cells. We analyzed K^+- and corticotrophin releasing factor-evoked hormone secretion from these cell lines by radioimmunoassay. We showed that expression of wild type GAP-43 in AtT-20 enhanced K^+-evoked secretion and caused the cells to flatten and elongate processes when plated on laminin-coated substrate. The domains of GAP-43 important for these effects were studied by investigating the action of GAP-43 mutated for CaM binding or membrane binding on hormone secretion and morphology in the AtT-20

cell culture model system. We showed that in AtT-20 cells expressing wild type GAP-43, or in hippocampal neurons, GAP-43 is associated with CaM and this interaction is regulated by intracellular Ca^{2+} concentration or phsophorylation. Furthermore, mutant forms of GAP-43 that cannot bind CaM or associate with the plasma membrane is unable to enhance K^+-evoked secretion. Thus, the binding of CaM to GAP-43 and the localization of GAP-43 at the plasma membrane are critical for GAP-43 function in the modulation of secretion. In contrast, the effect of GAP-43 in the morphological changes appear to be dependent only on the association of GAP-43 with the plasma membrane.

INTRODUCTION

During development of the nervous system, precursor cells migrate, differentiate into neurons, which extend axons and dendrites to establish connections with other parts of the nervous system or leave the nervous system to innervate effector tissues. It was the study of the process of axonal growth and synapse formation that led to the identification of "growth associated proteins". The genes for these proteins were shown to be activated transiently during the period of axon elongation either during development or in the case of successful nerve regeneration (reviewed by Benowitz and Routtenberg, 1987 ; Skene, 1989). The most intriguing of these proteins, which is rapidly transported to the growth cone and is concentrated at the inner face of the plasma membrane, is the growth associated protein (GAP)-43. GAP-43 has been studied for more than a decade and is thought to be implicated in different processes such as axonal growth, long-term potentiation or neurotransmitter release. However, the precise function of GAP-43 in these processes remains unclear.

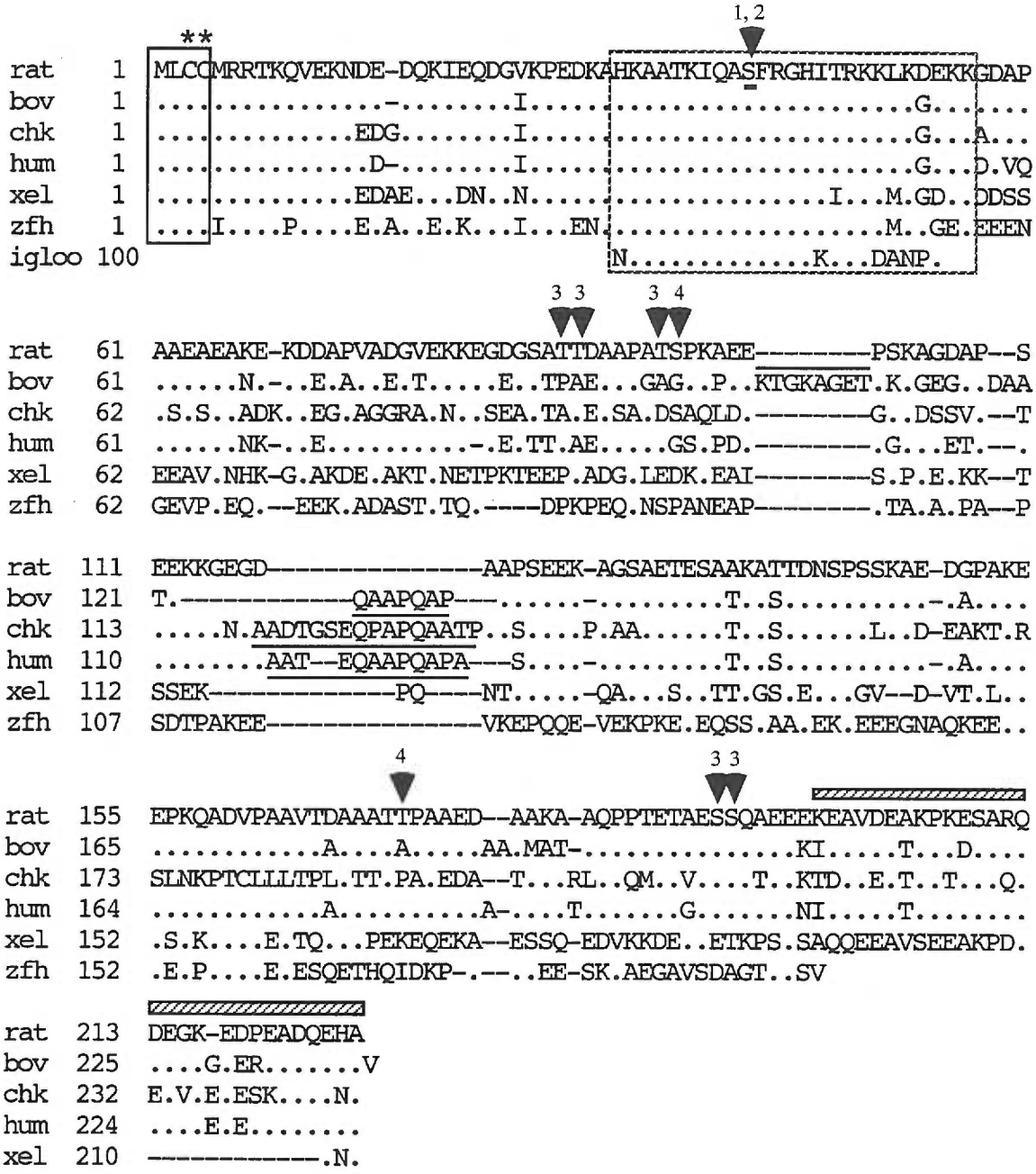
A / Primary Structure of GAP-43

GAP-43 is a membrane-bound phosphoprotein that has been discovered on several separate occasions, depending on the context where it was studied. The protein has been found to be expressed at a high level in the tip of the growing axon during development or nerve regeneration (reviewed in Benowitz and Routtenberg, 1987 ; Skene, 1989), hence its designation as

"growth-associated protein". Its apparent molecular weight, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), varies between 43 and 57 kDa, depending on the concentration of acrylamide in the gel (Benowitz *et al.*, 1987). This was the basis for many numerical designations assigned to the protein (P-57, GAP-43, GAP-48, B-50, pp46 ; reviewed by Benowitz and Routtenberg, 1987). In fact, the molecular weight of GAP-43, predicted from its amino-acid sequence, is about 23-25 kDa. Immunological characterizations (Gispen *et al.*, 1986 ; Meiri *et al.*, 1986 ; Snipes *et al.*, 1987) and the cloning of its cDNA from different species (Basi *et al.*, 1987 ; Cimler *et al.*, 1987 ; Karns *et al.*, 1987 ; Rosenthal *et al.*, 1987 ; Kosik *et al.*, 1988 ; Ng *et al.*, 1988 ; Baizer *et al.*, 1990) has revealed that these proteins are identical and that the GAP-43 gene is highly conserved in vertebrate evolution (La Bate and Skene, 1989).

The alignment of the amino acid sequences of GAP-43 from different species, shown in figure 1, demonstrates this high degree of conservation, particularly for the 60 N-terminal amino acids and to some extent in the C-terminus. The 10 N-terminal amino acids, constituting the membrane association domain, are identical in the mouse, chicken, bovine and human protein (Baizer *et al.*, 1990 ; figure 1). The next 50 amino acids again are highly conserved (90 % identity) and contain the phosphorylation site for the Ca^{2+}/phospholipid-dependent protein kinase (PKC) and the calmodulin-binding domain, (Baizer *et al.*, 1990 ; figure 1). The middle portion of the molecule, in contrast, is less conserved from one species to another.

Depending on the species, GAP-43 is made of 213 to 246 amino acids. The primary sequence indicates that the protein is acidic (pI = 4.3-4.5 ; Changelian *et al.*, 1990) and highly charged (23 % of aspartate or glutamate and 14 % of lysine or arginine ; figure 1).



(Adapted from (Skene, 1989 ; Baizer *et al.*, 1990)

Figure 1 : Alignment of the rat, bovine (*bov*), chicken (*chk*), human (*hum*), xenopus laevis (*xel*) and zebra fish (*zfh*) amino acid sequences of GAP-43. *Dots* indicate amino acids identical to the rat sequence. The *plain box* indicates the membrane binding domain ; the palmitylation sites are indicated by *asterisks*. The *dotted box* indicates the CaM-binding domain. *Arrow heads* indicate phosphorylation sites by 1) PKC, 2) phosphorylase kinase, 3) casein kinase II, 4) unidentified kinase ; Ser^{41}, the PKC phosphorylation site is underlined (*heavy*). Additional amino acid sequences in bovine, chicken and human are underlined (*thin*). *Dashes* in the sequence indicate spaces inserted to optimize alignment. *Shaded bars* indicate the putative neurofilament homology domain.

This may explain the anomalous migration of GAP-43 on SDS-PAGE : the highly charged nature of this polypeptide may prevent it to bind SDS efficiently, resulting in a delayed migration.

Biophysical analyses performed on the bovine GAP-43 by sucrose gradient density sedimentation and fluorescence polarization indicated that the molecule is elongated (axial ratio of 16:1 ; Masure *et al.*, 1986). Proton nuclear magnetic resonance (NMR) and circular dichroism studies revealed that the secondary structure of the protein is mainly random coil (78 %) with very little organized secondary structure (1 % alpha-helix, 21 % β-sheet ; Masure *et al.*, 1986 ; Coggins and Zwiers, 1989). In addition of the presence of numerous charged side chains which may produce repulsive forces and restrict the ability of the polypeptide to fold back on itself, the abundance of proline residues, particularly in the C-terminal region (up to 16 % of the residues), contributes to rigidity of the protein.

It has been suggested that exons define separate domains in a protein. Thus, in the GAP-43 gene, the membrane association domain is encoded by exon 1 (10 N-terminal amino acids), exon 2 (189-210 aa, depending on the species) encodes the CaM-binding domain and exon 3 (28-29 C-terminal amino acids) the putative cytoskeleton-binding domain, as discussed below.

B / Functional domains of GAP-43 :

1. Membrane association domain:

GAP-43 is a membrane associated protein (Cimler *et al.*, 1985 ; Skene *et al.*, 1986 ; Dosemeci and Rodnight, 1987 ; Gorgels *et al.*, 1989 ; Van Hoof *et al.*, 1989b ; Houbre *et al.*, 1991), despite the lack of a transmembrane domain. The N-terminal sequence is thought to be responsible for the membrane targeting of GAP-43 (figure 1 and Liu *et al.*, 1993). Chimeras obtained by fusing soluble

proteins with the N-terminal domain of GAP-43 have been found to be membrane associated (Zuber *et al.*, 1989b ; Liu *et al.*, 1991).

Mutation of the cysteines 3 & 4 prevented the association of GAP-43 with the plasma membrane in transfected cells (Zuber *et al.*, 1989b ; Liu *et al.*, 1991 ; Liu *et al.*, 1993 ; Liu *et al.*, 1994). The attachment of GAP-43 to the membrane involves posttranslational palmitylation (Skene and Virág, 1989 ; Chapman *et al.*, 1992 ; Liu *et al.*, 1993). Palmitate can be linked reversibly to two cysteine residues in the N-terminus (cysteine 3 and 4) through thioester bonds (Sudo *et al.*, 1992), with cys^4 being critical for palmitate incorporation and membrane association, since substitution of this residue by leucine resulted in 75 % reduction in palmitate incorporation compared with the wild type GAP-43 (Liu *et al.*, 1993). The intercalation of fatty acid chains into the lipid bilayer allows GAP-43 to bind non specifically to the inner face of the plasma membrane. Although GAP-43 is mostly membrane bound, a small amount of the protein is also present in the soluble fraction, since GAP-43 is synthesized in the cytosol by free ribosomes. The palmitylation of GAP-43 is a posttranslational modification and is critical for the packaging of GAP-43 in the Golgi and delivery to the growth cone by fast axonal transport (Goslin *et al.*, 1990 ; Liu *et al.*, 1994).

In addition to the critical 2 N-terminal amino acids, other amino acids in the N-terminal domain of GAP-43 may contribute to its association with the plasma membrane, since GAP-43 or a peptide corresponding to residues 37-53 can bind to phospholipid vesicles (Houbre *et al.*, 1991 ; Kim *et al.*, 1994). A fusion protein between the N-terminal 10 amino acids of GAP-43 and chloramphenicol acetyltransferase (CAT) accumulated in the growth cone of PC12 cells (Zuber *et al.*, 1989b). However D. Storm's collaborators found that other structural elements of GAP-43 may be required for growth cone

targeting, since a fusion protein between β-galactosidase and GAP-43 or its N-terminal 20 amino acids could be transported to the growth cone of cultured rat neurons, but not a fusion protein containing only the 10 N-terminal amino acids of GAP-43 and β-galactosidase (Liu *et al.*, 1991 ; Liu *et al.*, 1994). Phosphorylation of GAP-43 or GAP-43 (37-53) by PKC reduced the binding to phospholipid vesicles (Houbre *et al.*, 1991 ; Kim *et al.*, 1994), suggesting that GAP-43 association with the plasma membrane may be a dynamic process. In PC12 cells, NGF treatment induces translocation of GAP-43 from the Golgi apparatus to the plasma membrane (Van Hoof *et al.*, 1989b).

These results suggest that the dynamic association of GAP-43 with the plasma membrane may be tightly regulated and important for the function of the protein in the growth cone.

2. Interaction of GAP-43 with heterotrimeric GTP-binding proteins :

A short sequence in the amino terminus of GAP-43 resembles cytoplasmic regions of G-coupled receptors which are known to interact with trimeric GTP-binding proteins ("G-proteins"). As G_o, a member of the G-protein family, was shown to be very abundant in the growth cone, its interaction with GAP-43 was investigated (Strittmatter *et al.*, 1990). The N-terminal domain of GAP-43 (10 first amino acids) can stimulate GTP/GDP exchange on the α subunit of G_o or G_i (Strittmatter *et al.*, 1990 ; Strittmatter *et al.*, 1991 ; Strittmatter *et al.*, 1994a).

The cysteine residues also appear to be crucial for GAP-43 interaction with G-proteins since replacement of cys^3 and cys^4 by threonine residues abolishes this activity (Strittmatter *et al.*, 1990). Palmitylation of these 2 cysteines also blocks the stimulation of G-proteins (Sudo *et al.*, 1992).

While the N-terminal domain of GAP-43 shares sequence homologies with a number of G-protein-linked transmembrane receptors (Strittmatter *et al.*, 1990), GAP-43 appears to activates G_o or G_i through a different mechanism. Unlike the case for receptors, the effect of GAP-43 was not inhibited by pertussis toxin, nor affected by the presence or absence of $\beta\gamma$ subunits or phospholipids (Strittmatter *et al.*, 1991). Injection of purified GAP-43 into *Xenopus laevis* oocytes or dorsal root ganglion (DRG) neurons enhanced G-protein-dependent signal transduction cascade (Strittmatter *et al.*, 1993 ; Igarashi *et al.*, 1995).

3. Phosphorylation of GAP-43 :

• *Phosphorylation of GAP-43 by the Ca^{2+}/phospholipids-dependent protein kinase (PKC):*

In the growth cone GAP-43 has been shown to be a major substrate for PKC (Akers and Routtenberg, 1985 ; de Graan *et al.*, 1985 ; Meiri *et al.*, 1986 ; Skene *et al.*, 1986 ; Nelson *et al.*, 1989). GAP-43 is phosphorylated by PKC on serine 41 (Coggins and Zwiers, 1989 ; Apel *et al.*, 1990). This residue is part of the calmodulin-binding domain (Alexander *et al.*, 1988), a positively charged region which includes the only aromatic residue of the protein (phe^{42} ; figure 1 and below). Phosphorylation of ser^{41} by PKC, or its replacement by an aspartate residue, introduces a negative charge which disrupts the interaction between GAP-43 and calmodulin (Chapman *et al.*, 1991). However, GAP-43 bound to calmodulin cannot be phosphorylated by PKC (Chan *et al.*, 1986 ; Alexander *et al.*, 1987).

Phosphorylation of GAP-43 by PKC has been shown to be dependent on extracellular calcium (Dekker *et al.*, 1989 ; Dekker *et al.*, 1990 ; Heemskerk *et al.*, 1990 ; Schaechter and Benowitz, 1993), when stimulated by depolarization,

activation of muscarinic receptors, nerve growth factor or by arachidonic acid (Dekker *et al.*, 1989 ; Van Hoof *et al.*, 1989a ; Dekker *et al.*, 1990 ; Meiri and Burdick, 1991 ; Schaechter and Benowitz, 1993). The degree of GAP-43 phosphorylation by PKC is highest in regions of the brain involved in the storage of visual representation (Nelson *et al.*, 1987) or LTP (Lovinger *et al.*, 1985 ; Chan *et al.*, 1986 ; Gianotti *et al.*, 1992) and in the distal axon but not in the cell bodies of neurons of the olfactory projection (Meiri *et al.*, 1991). These results suggest that phosphorylation of GAP-43 by PKC is involved in signal transduction in nerve terminals.

• *Phosphorylation of GAP-43 by other kinases, additional sites of phosphorylation and dephosphorylation :*

GAP-43 contains several serine and threonine residues, potential sites for phosphorylation (figure 1). Analysis by two-dimensional electrophoresis revealed that GAP-43 was phosphorylated at multiple sites (Zwiers *et al.*, 1985). GAP-43 has been shown to be a substrate for casein kinase II. *In vitro* casein kinase II phosphorylates mouse GAP-43 at ser^{192}, ser^{193}, thr^{88}, thr^{89} and thr^{95} (Apel *et al.*, 1991 ; Spencer *et al.*, 1992). Phosphorylation of GAP-43 by casein kinase II is also inhibited by the binding of calmodulin to GAP-43 (Apel *et al.*, 1991).

In cultured neurons and rat brain, ser^{96} and thr^{172} were phosphorylated, but the protein kinase responsible was not identified (Spencer *et al.*, 1992). In addition, bovine GAP-43 is a substrate for phosphorylase kinase, which phosphorylates GAP-43 at ser^{41} (Paudel *et al.*, 1993).

However, the physiological significance of these other phosphorylation sites remains unclear, only phosphorylation of GAP-43 by PKC has been correlated with physiological changes.

GAP-43 is a substrate for the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Liu and Storm, 1989), but the dephosphorylation of GAP-43 has been little studied and its physiological significance is unclear (Liu and Storm, 1989 ; Schrama *et al.*, 1989 ; Dokas *et al.*, 1990).

4. Interaction of GAP-43 with calmodulin :

D. Storm's group was studying calmodulin-binding proteins and originally isolated bovine GAP-43 (designated as P-57 and later neuromodulin) as a protein which binding to calmodulin (CaM) was unusually affected by calcium concentration. In contrast to most other calmodulin-binding proteins, the affinity of GAP-43 for calmodulin is higher at a low calcium concentration than at high calcium (Andreasen *et al.*, 1981 ; Andreasen *et al.*, 1983 ; Cimler *et al.*, 1985).

The association between GAP-43 and calmodulin was studied *in vitro* by fluorescence anisotropy measurements, using 5-[[(iodoacetylamino)ethyl]-amino]-1-naphtalenesulfonic acid-labeled calmodulin (AEDANS-CaM)(Alexander *et al.*, 1987). Under low ionic strength conditions, the dissociation constants for the AEDANS-CaM-GAP-43 complex are 2.3×10^{-7} M in the absence of calcium and 10^{-6} M in the presence of 1 mM calcium chloride.. Furthermore, the interaction between GAP-43 and CaM depends on ionic strength conditions since in the presence of 150 mM KCl the Ca^{2+}-independent and -dependent dissociation constants were increased to 3.4×10^{-6} M and 3×10^{-6} M respectively (Alexander *et al.*, 1987).

The CaM-binding domain of GAP-43 was subsequently identified as a 26 amino acids sequence QASFRGHITRKKLKGEKK (residues 32-56 of the bovine GAP-43 ; figure 1) and forms an α-helix (Alexander *et al.*, 1988 ; Chapman *et al.*, 1991 ; Gerendasy *et al.*, 1995). This domain contains the only

phenylalanine residue of GAP-43 (phe^{42}), which is critical for the binding of CaM to GAP-43 since its replacement by a tryptophan residue increased the affinity of CaM for GAP-43. Another critical residue is serine-41, the phosphorylation site for PKC. Phosphorylation of this residue by PKC or its substitution of by an aspartic acid, both of which modifications introduce a negative charge in the molecule, abolished the binding of CaM to GAP-43 (Alexander *et al.*, 1987 ; Chapman *et al.*, 1991).

These results suggest that in unstimulated cells, CaM would be complexed to GAP-43 and led D. Storm to propose that the function of GAP-43 is to sequester calmodulin at the inner face of the plasma membrane in the vicinity of calmodulin-activated enzymes under low calcium conditions. Elevation of intracellular calcium would promote dissociation of calmodulin from GAP-43, allowing rapid activation of calcium/calmodulin-dependent processes (Alexander *et al.*, 1987 ; Apel and Storm, 1992).

Other investigators have suggested that GAP-43 may also serve as a carrier for CaM : the binding of CaM, a slowly transported protein, to GAP-43 would permit its rapid delivery to the growth cone plasma membrane by fast axonal transport (Spencer and Willard, 1992).

A GAP-43 homologue recently cloned from Drosophila, *igloo* (Neel and Young, 1994), which has been shown to interact with Drosophila CaM, has very little sequence homology with GAP-43 from other species except for a 23 amino acid sequence 70 % identical to the CaM-binding domain (figure 1).

5. Interaction of GAP-43 with the cytoskeleton.:

The elongated shape of GAP-43, as suggested by its characteristics described earlier, would allow GAP-43 to extend from the plasma membrane and make contact with the subjacent cytoskeleton. In addition to its membrane binding

through palmitylation, GAP-43 also appears to be interacting with the membrane skeleton (Allsopp and Moss, 1989 ; Meiri and Gordon-Weeks, 1990; Moss *et al.*, 1990), an actin-rich network cross-linked with other proteins which is responsible for cell shape. GAP-43 has been shown to bind filamentous actin in a saturable fashion, with a K_d of 4 μM (Strittmatter *et al.*, 1992). The C-terminal domain of GAP-43 is thought to be involved in this interaction, which may localize GAP-43 at specific sites of the plasma membrane. A sequence in the C-terminal domain of GAP-43 shares homology with a portion of neurofilament NF-L putatively mediating interaction with the cytoskeleton (La Bate and Skene, 1989). Alternatively, since actin polymerization/depolymerization cycles are responsible for movements of the leading edge of the growth cone, GAP-43 may regulate these movements. In primary sensory neurons depleted of GAP-43 by antisense GAP-43 oligonucleotides, the lamellar extensions of the growth cone lacked local f-actin concentrations and showed poor adhesion (Aigner and Caroni, 1995). It has been proposed that this interaction with actin could be regulated by phosphorylation of residues in the C-terminal region of GAP-43, for example by casein kinase II, as discussed earlier.

These characteristics of GAP-43 suggest that the protein is a common mediator of several second messenger pathways and in a position to regulate several aspects of axon elongation, synapse formation and function. Indeed GAP-43 is thought to be involved in all of these processes.

C/ Function of GAP-43 :

1. Function in neurite extension :

- *The growth cone and axonal elongation* :

Neurons are polarized cells. This polarity develops when one of the many short processes originally extended by the unpolarized neuron grows more rapidly and acquires axonal characteristics. At the tip of the axon, the structure where the process of membrane addition for the growth of the axon is taking place is called the growth cone. Axons elongate along a precise path, in response to signals from the environment. Thus, the function of the growth cone is also to interpret extracellular signals.

Growth cones have numerous extensions, some of them broad called lamellipodia, some of them fine and designated as filopodia, constantly extending and retracting. At the growing tip of these extensions, membrane vesicles originating from the Golgi and transported along the axon fuse with the plasma membrane. Major components of the filopodia and lamellipodia are the actin microfilaments, which are responsible for movements of these extensions.

Actin polymerizes *in vivo* by addition of actin monomers at the membrane-associated barbed end, thus causing translocation of the actin filament toward the cell body. Myosin is also involved in the movement of actin by interacting with the actin microfilaments and providing retrograde power accompanied by ATP hydrolysis. To modulate advance or retraction of the filopodia, the polymerization/depolymerization cycles of actin are also controlled by receptors upon binding to their ligand. This process underlies the orientation phase, in response to an interaction of the growth cone to guidance cues. Once the direction is determined, microtubules are

polymerized in the direction of growth during a phase of consolidation. Finally, at the neck of the growth cone, microtubules and actin are converted into stable forms and the axon tube is formed during the conversion phase (Mitchison and Kirschner, 1988)

- *Expression of GAP-43 during the development of the nervous system :*

GAP-43 is a neural specific protein, very abundant in the brain where it constitutes 0.1 % of the total proteins of the white matter (reviewed in Benowitz and Routtenberg, 1987 ; Gordon-Weeks, 1989 ; Skene, 1989). During development, GAP-43 is epxpressed in all neurons of the peripheral and central nervous system at the time when neurons are extending axons (Skene and Willard, 1981a ; Jacobson *et al.*, 1986 ; Kalil and Skene, 1986 ; Skene *et al.*, 1986 ; Basi *et al.*, 1987 ; Moya *et al.*, 1987 ; Allsopp and Moss, 1989 ; de La Monte *et al.*, 1989 ; Biffo *et al.*, 1990). In the hamster pyramidal tract neurons, a 43K protein, which resembles GAP-43, is transported into growing axons during the first 2 weeks of postnatal development, and its dramatic decline by the fourth postnatal week coincides with the cessation of axonal elongation (Kalil and Skene, 1986). In the chicken embryo a protein very similar to GAP-43, designated as 3D5 antigen, is found in the CNS and the PNS with maximum expression occurring at E14-E16 and decreasing 4-fold in the adult brain (Allsopp and Moss, 1989). Immunocytochemistry and *in situ* hybridization experiments showed that GAP-43 was expressed in all neurons during the perinatal period and then disappeared from most neurons as they matured (de La Monte *et al.*, 1989). Thus GAP-43 is synthesized in the cell body of developing neurons, then is rapidly transported along the axon (within hours of its synthesis) and concentrates in the growth cone of elongating axons (de Graan *et al.*, 1985 ; Meiri *et al.*, 1986 ; Skene *et al.*, 1986 ; Goslin *et al.*, 1988 ;

Allsopp and Moss, 1989 ; Gorgels *et al.*, 1989 ; Goslin *et al.*, 1990 ; Van Lookeren Campagne *et al.*, 1990) where it constitutes up to 1 % of the membrane proteins. GAP-43 is found in clusters at the plasma membrane (Meiri *et al.*, 1986 ; Meiri and Gordon-Weeks, 1990), suggesting that it is not inserted at random sites, but may be targeted at specific sites, possibly through interactions with the membrane skeleton, as discussed previously.

Although GAP-43 is thought to be primarily a neuronal protein, there has been several reports that GAP-43 may also be expressed in non neuronal cells, particularly glial cells, or supporting cells of the nervous system. GAP-43 is expressed in astrocytes of type I and type II and oligodendrocytes, which are macroglial cells. GAP-43 expression was constitutive in type II astrocytes and oligodendrocytes, but developmentally regulated in type I astrocytes (Vitkovic *et al.*, 1988 ; da Cunha and Vitkovic, 1990). GAP-43 has also been shown to be expressed by non-myelin-forming Schwann cells (Curtis *et al.*, 1992). Finally, GAP-43 has been shown to be expressed in non-neuronal cells of the embryonic chicken limb (Stocker *et al.*, 1992). The role of GAP-43 in these non-neuronal cells is unclear, but GAP-43 may be involved in fusion events similar to those that occur during exocytosis or membrane addition.

- *Expression of GAP-43 during regeneration of the nervous system :*

The expression of GAP-43 declines after the completion of synaptogenesis (de La Monte *et al.*, 1989), but is increased again in the case of successful nerve regeneration following injury in those organisms that retain the ability of central nervous system (CNS) regeneration, or in the peripheral nervous system (PNS) of all vertebrates (Skene, 1984). GAP-43 is reexpressed following axotomy of peripheral nerve in the rabbit but not in the CNS, which does not regenerate (Skene and Willard, 1981a). In a number of studies where GAP-43

expression has been examined after nerve injury, GAP-43 expression dramatically increased in correlation with regeneration following injury : in the optic nerve of the toad (Skene and Willard, 1981b) and of the goldfish (Benowitz *et al.*, 1981 ; Benowitz and Lewis, 1983 ; Perry *et al.*, 1987) ; in the DRG and in the regenerating sciatic nerve after sciatic nerve crushing in adult rats (Tetzlaff *et al.*, 1989 ; Woolf *et al.*, 1990). In the olfactory system, after a lesion the expression of GAP-43 increased during regeneration then decreased as the olfactory neuron matured when reaching their target. However, if the developing neurons did not have access to their target during regeneration, they failed to mature and their GAP-43 levels remained elevated (Verhaagen *et al.*, 1990).

Overexpression of GAP-43 in the nervous system of transgenic mice caused spontaneous nerve sprouting at the neuromuscular junction and potentiated lesion-induced nerve sprouting and terminal arborization during reinnervation (Aigner *et al.*, 1995). These results suggest that GAP-43 may be necessary for axon elongation and that the lack of GAP-43 expression may be one of the reasons for the failure of CNS nerves to regenerate.

- *Expression of GAP-43 in cultured cell model systems :*

The correlation between the level of GAP-43 expression and neurite elongation has also been studied in tissue culture. Using cultures of cerebellar granule cells, it was possible to reproduce the observations made *in vivo*, that GAP-43 expression was correlated with axonal elongation and then decreased with neuronal maturation. In these cells, one day after plating GAP-43 was expressed in cell bodies as well as in neurites. GAP-43 subsequently disappeared from the cell body to concentrate in the growth cone. By 20 days the expression of GAP-43 had declined in the growth cone, in contrast to that

of p65, a synaptic vesicle protein, which expression increased from 3 to 20 days in culture, as neurons matured (Burry *et al.*, 1991).

Treatment of PC12 pheochromocytoma cells with nerve growth factor (NGF) causes the expression of neuron specific characteristics (including GAP-43) and extension of processes. Transfection of the human GAP-43 cDNA into PC12 cells enhanced neurite outgrowth in response to NGF (Yankner *et al.*, 1990). Transfection of GAP-43 into NG108-15 cells caused neurite formation (Kumagai *et al.*, 1992 ; Kumagai-Tohda *et al.*, 1993).

Blocking the expression of GAP-43 by antisense GAP-43 oligonucleotides decreased neurite extension, spreading, branching and adhesion from cultured DRG neurons (Aigner and Caroni, 1993 ; Aigner and Caroni, 1995). Introduction of anti-GAP-43 antibodies in neuroblastoma cells decreases neurite formation (Shea *et al.*, 1991). The expression of GAP-43 in non-neuronal cell lines also appears to cause filopodia formation (Zuber *et al.*, 1989a ; Widmer and Caroni, 1993 ; Strittmatter *et al.*, 1994b). Again, these results suggest that GAP-43 is involved in neurite extension.

However, GAP-43 does not seem to be essential for the elongation of processes, since PC12 cells lacking or with reduced GAP-43 can still grow neurites in response to a treatment with NGF (Baetge and Hammang, 1991 ; Baetge *et al.*, 1992 ; Neve *et al.*, 1992 ; Aigner and Caroni, 1993 ; Widmer and Caroni, 1993) and the extent of the neurite outgrowth did not correlate with the levels of GAP-43 in several clonal PC12 cell lines (Burry and Perrone-Bizzozero, 1993). GAP-43 is absent from growing dendrites or dendritic growth cones of hippocampal neurons in culture (Goslin *et al.*, 1988). DRG neurons depleted of GAP-43 with antisense oligonucleotides cultured on laminin extended long neurites, however, when plated on poly-L-ornithine, these neurons failed to form growth cone and elongate processes (Aigner and

Caroni, 1993). Injection of GAP-43 or GAP-43 peptides in cultured DRG neurons, enhanced their response to growth-inhibitory ligands (Igarashi *et al.*, 1993). Furthermore, GAP-43 gene knockout mice had normal nerve growth rates and normal CNS. Additionally, cultured neurons derived from these mice extended neurites normally. However, their retinal axons lacking GAP-43 could not pass the midline decision point in the optic chiasm, suggesting that their growth cones failed to interpret axonal guidance cues from their environment (Strittmatter *et al.*, 1995).

Taken together, these results suggest that GAP-43 is not an essential structural component of the growth cone necessary for axon elongation, but rather might serve as a common mediator of several signal transduction pathways modulates extracellular signals sent by the target for innervation to direct and control axonal growth. However, the precise mechanism of action of GAP-43 in the modulation of the extracellular signal is still unclear.

Intriguingly, transgenic Drosophila embryo axons in which CaM was inhibited by a specific CaM antagonist stalled and exhibited abnormal crossing of the midline (VanBerkum and Goodman, 1995), similar to errors in axon guidance described in GAP-43 knock-out transgenic mice (Strittmatter *et al.*, 1995), suggesting an interaction between GAP-43 and CaM may play a role in axon guidance.

2. Role of GAP-43 in synaptic function :

- *The presynaptic terminal :*

When the axon reaches its target and establishes the synapse, the growth cone then turns into a presynaptic terminal, which is also a dynamic structure where neurotransmitters are released to transmit the information to the

postsynaptic neuron or effector. The major organelles of the synaptic region are synaptic vesicles, which accumulate, store and release neurotransmitter.

The vesicles containing the neurotransmitter and the components of the synaptic vesicles are synthesized in the cell body and transported rapidly to the nerve terminal. Some of these vesicles, forming the storage pool of vesicles, are associated with the actin filament in the nerve terminal. These vesicles are cross-linked to each other and anchored to the cytoskeleton through synapsin, a family of vesicle-associated proteins. Synapsin I is a substrate for the calcium/calmodulin-dependent kinase II and the phosphorylation of synapsin I reduces its affinity for synaptic vesicles andits ability to bundle f-actin. This suggests that the phosphorylation of synapsin I by CaM-kinase II upon an influx of calcium in the nerve terminal could regulate the mobilization of synaptic vesicles.

Mobilized vesicles accumulate at specialized sites of the plasma membrane designated as the active zone, where a network of actin filaments closely associated with the active zone hold the docked vesicles at approximately 30 nm from the plasma membrane. These docked vesicles form the releasable pool of synaptic vesicles and fuse with the plasma membrane only after an appropriate stimulus.

Elevation of intracellular calcium is considered to be the trigger for regulated secretion of neurotransmitter by nerve cells. The fusion of vesicle membrane with the plasma membrane is thought to involve a complex of proteins from the vesicle and the plasma membrane, phospholipids and calcium. However, the biochemical events responsible for transmission of the calcium signal into vesicle fusion and exocytosis are not very well understood (Augustine *et al.*, 1987 ; Trimble *et al.*, 1991 ; Burns and Augustine, 1995). This is the subject of very active research and a number of proteins involved in

vesicle docking and fusion have been identified, but their precise function is still debated (Alder and Poo, 1993 ; Bennett and Scheller, 1993 ; Walch-Solimena *et al.*, 1993).

This complex process can be regulated with respect to the amount of product released, as well as its timing. For example, there is evidence that during long-term potentiation, the amount of neurotransmitter released by the presynaptic terminal is increased (Bekkers and Stevens, 1990 ; Malinow and Tsien, 1990). Calcium mobilization can vary depending on the stimulus and the signal transduction pathway it activates. Phosphorylations and G-proteins can regulate receptor affinity as well as opening of ion channels, protein-protein interactions and alterations of the cytoskeleton, which can affect vesicle movements and fusion with the plasma membrane (Trimble *et al.*, 1991).

- *Expression of GAP-43 in the adult nervous system :*

Upon maturation of the neuron, the growth cone turns into a presynaptic terminal and GAP-43 levels decrease dramatically (Verhaagen *et al.*, 1990 ; Burry *et al.*, 1991). In discrete regions of the adult nervous system, however GAP-43 levels remain elevated. These regions are associated with learning and memory, where synaptic remodeling and strengthening, or synaptic plasticity, is thought to occur (Gispen *et al.*, 1985 ; Neve *et al.*, 1987 ; Benowitz *et al.*, 1988 ; Neve *et al.*, 1988 ; Benowitz *et al.*, 1989 ; Masliah *et al.*, 1991).

In the adult human cerebral cortex, GAP-43 expression was low in primary sensory and motor areas and higher in associative regions (Neve *et al.*, 1988 ; Benowitz *et al.*, 1989). GAP-43 expression is found to be moderately high in the adult entorhinal cortex and strikingly high in the adult hippocampus and olfactory bulb (de La Monte *et al.*, 1989). In adult rat brain, the highest GAP-43

mRNA level was found in the hippocampus, particularly in the CA1-3 region where it overlapped with that of CaM-kinase II, which has been linked to synaptic plasticity (Neve *et al.*, 1988 ; Jacobs *et al.*, 1993).

The phosphorylation of GAP-43 by PKC has been found to be elevated in visual memory storage areas (Nelson *et al.*, 1987), as well as correlated with long term potentiation (LTP)a model for synaptic plasticity (Lovinger *et al.*, 1985 ; Routtenberg *et al.*, 1985 ; Chan *et al.*, 1986 ; Gianotti *et al.*, 1992) and neurotransmitter release (Dekker *et al.*, 1989). In rat hippocampal slices, phosphorylation of GAP-43 was increased up to 70 % from 10 to 60 min after long-term potentiation had been induced, and this increase was not observed when long term potentiation failed (Ramakers *et al.*, 1995). In isolated synaptosomes, phosphorylation of GAP-43 by PKC can be induced by depolarization (Dekker *et al.*, 1990 ; Heemskerk *et al.*, 1990), suggesting a possible role for GAP-43 in synaptic plasticity.

- *Role of GAP-43 in neurotransmitter release :*

The involvement of GAP-43 in secretion has been addressed by different approaches. Isolated synaptosomes were detergent-permeabilized, to allow uptake of antibodies or elevation of intracellular calcium. In this system, anti-GAP-43 antibodies that prevent its phosphorylation by PKC, inhibit evoked neurotransmitter release from small synaptic vesicles, as well as peptide-containing, large dense-cored vesicles (Dekker *et al.*, 1989 ; Hens *et al.*, 1993b ; Hens *et al.*, 1995). Potassium-depolarization of synaptosomes, which induces neurotransmitter release, induced a transient. enhancement of GAP-43 phosphorylation (Dekker *et al.*, 1990). However, in this system, when GAP-43 phosphorylation by PKC was blocked by specific PKC inhibitors, the release of neurotransmitter could still be triggered by an elevation of calcium (Dekker *et*

al., 1991 ; Hens *et al.*, 1993a ; Hens *et al.*, 1993b), suggesting that phosphorylation of GAP-43 by PKC may not be necessary for GAP-43 function in neurotransmitter release. Other investigators have used cultured cell lines which express GAP-43 and in which its expression can be modulated. For example, in NG108-15 cells a correlation between the GAP-43 mRNA levels and the neurotransmitter release level was observed (Kumagai-Tohda *et al.*, 1993). In PC12 cells, inhibition of GAP-43 synthesis by antisense RNA blocked the secretion of noradrenaline in response to an elevation of intracellular calcium (Neve *et al.*, 1992 ; Ivins *et al.*, 1993).

GAP-43 also appears to be involved in the modulation of calcium mobilization, which could in turn influence exocytosis. It was reported that in PC12 cells GAP-43 modulates the function of L-type calcium channels, thereby affecting their electrical excitability (Baetge *et al.*, 1992). Additionally, as an inhibitor of the phosphatidylinositol 4-phosphate kinase (PIP) kinase, GAP-43 influences the metabolism of phosphoinositides and may act as a feedback inhibitor of calcium mobilization and PKC activity (Jolles *et al.*, 1980). More recently, GAP-43 was shown to be ADP-ribosylated (Coggins *et al.*, 1993), however the functional significance of this is unknown.

These results support the notion that GAP-43 is involved in the regulation of exocytosis of secretory vesicles (neurotransmitters) as well as that of dense-core granules (peptides). GAP-43 may also modulate vesicle-membrane fusion during the process of neurite extension, but despite intense efforts these studies have still yielded little information on the precise mechanism of action of GAP-43 in any of these processes.

D. Storm proposed that GAP-43, as it is an elongated hydrophilic molecule which extends from the plasma membrane to the membrane skeleton and

binds CaM, might act to localize and concentrate CaM near its sites of action. In this scenario, GAP-43 would release CaM upon an increase of intracellular calcium to enhance calcium/calmodulin-dependent processes.

We have decided to test this hypothesis in a cell culture model system that would allow manipulations of the expression of GAP-43 and observation of the consequences on a physiological function of the cell.

The establishment of such a model system is reported in the first manuscript, which describes how GAP-43 when expressed in the mouse anterior pituitary tumor cells AtT-20 enhanced K^+-evoked exocytosis.

This model system was then used to demonstrate that the interaction between CaM and GAP-43 was regulated by calcium and that the binding of CaM to GAP-43, as well as the membrane localization of GAP-43 were essential to the function of GAP-43 in K^+-evoked exocytosis. These experiments are reported in the second manuscript.

MANUSCRIPT #1

Growth-associated protein-43 (GAP-43) facilitates peptide hormone secretion in mouse anterior pituitary AtT-20 cells

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The abbreviations used are :
GAP, growth-associated protein ; PKC, Ca^{2+}/phospholipid-dependent protein kinase ; CRF, corticotropin-releasing factor ; RSV, Rous sarcoma virus ; nt, nucleotide ; PIPES, 1,4-piperazinediethanesulfonic acid

Summary

The neuronal growth-associated protein (GAP)-43 (neuromodulin, B-50, F1), which is concentrated in the growth cones of elongating axons during neuronal development and in nerve terminals in restricted regions of the adult nervous system, has been implicated in the release of neurotransmitter. To study the role of GAP-43 in evoked secretion, we transfected mouse anterior pituitary AtT-20 cells with the rat GAP-43 cDNA and derived stably transfected cell lines. Depolarization-mediated β–endorphin secretion was greatly enhanced in the GAP-43-expressing AtT-20 cells without a significant change in Ca^{2+} influx ; in contrast, expression of GAP-43 did not alter corticotropin-releasing factor-evoked hormone secretion. The transfected cells also displayed a flattened morphology and extended processes when plated on laminin-coated substrates. These results suggest that AtT-20 cells are a useful model system for further investigations on the precise biological function(s) of GAP-43.

INTRODUCTION

Growth associated (GAP)-43 (also known as B-50, F1, neuromodulin, P-57 and pp46) is a membrane-associated phosphoprotein expressed primarily in neurons (1-6). While its precise biological function remains to be determined, it is concentrated in the growth cone of developing neurons (7, 8) and expressed at elevated levels during periods of axonal growth and regeneration (for reviews see Refs. 9-12). Together with its association with the membrane skeleton (13, 14), this suggests that GAP-43 may be involved in the membrane addition associated with axonal elongation.

GAP-43 is a major neuronal calmodulin-binding protein that displays higher affinity for calmodulin in the absence of Ca^{2+} than in its presence (15-18). GAP-43 is also a prominent substrate of the calcium/phospholipid dependent protein kinase (PKC) (19-22) ; phosphorylation of GAP-43 by PKC decreases its affinity for calmodulin (16). Additionally, GAP-43 has been shown to interact with and activate GTP-binding, or 'G' proteins (23-26). Thus, GAP-43 appears to be a common mediator of several second messenger pathways and is therefore in a position to modulate the rate, extent or direction of axonal growth in response to external stimuli.

GAP-43 expression persists in regions of the mature nervous system that retain the potential for plasticity in response to neuronal activity (27-30). In the hippocampus, the correlation of PKC-mediated phosphorylation of GAP-43 with long-term potentiation (31, 32) suggests that GAP-43 may play a role in synaptic transmission. Support for this hypothesis has been provided by experiments demonstrating that *in vitro* phosphorylation of GAP-43 by PKC is correlated with potassium-evoked neurotransmitter release (33). Furthermore, introduction of antibodies which interfere with GAP-43

phosphorylation into permeabilized synaptosomes inhibits Ca^{2+}-induced neurotransmitter release (34-38). Finally, antisense RNA-mediated inhibition of GAP-43 in PC12 cells leads to a decreased release of dopamine in response to elevated potassium (39). These investigations suggest that GAP-43 is necessary for exocytosis but additional experiments are required to further define the role of GAP-43 in this process.

The AtT-20/D16-16 (D16) cell line is a subclone (40) of the original AtT-20 mouse pituitary tumor cell line (41). Both of these cell lines secrete pro-opiomelanocortin-derived peptide hormones (42, 43). The D16 cells are more amenable to manipulation in culture and respond to a variety of secretagogues, including corticotrophin releasing factor (CRF), noradrenaline, potassium, phorbol esters and calcium ionophores (reviewed in Ref. 44) and have therefore been used widely to study secretion (45). We have discovered that D16 cells express GAP-43 at high levels and display a robust secretory response to potassium-mediated membrane depolarization. In contrast, GAP-43 is undetectable in the original AtT-20 cells, and potassium evokes only a modest amount of hormone release. This correlation led us to investigate further the role of GAP-43 in neuropeptide secretion in the AtT-20 cell lines.

EXPERIMENTAL PROCEDURES

Plasmid construction. The expression vector for rat GAP-43 was produced by ligating a 1.1 kb restriction fragment containing the entire rat GAP-43 coding sequence (5) into the pRc/RSV vector (Invitrogen) with the Rous sarcoma virus (RSV) promoter (46) driving expression of the cDNA. To generate the probe for the RNase protection assay a 300 bp *Dra* I/*Sac* I restriction fragment derived from the rat GAP-43 expression vector (which includes a portion of 3'-untranslated region of the rat GAP-43 cDNA and some adjacent vector sequences) was ligated into pGEM-3Zf (Promega). This riboprobe will protect two fragments : the larger (approximately 270 nt) results from hybridization with the mRNA transcribed from the transfected rat GAP-43 cDNA, and the smaller fragment (approximately 240 nt) results from hybridization with the endogenous GAP-43 transcript.

Cell culture and transfections. All cell culture reagents were from Life Technologies, Inc. Monolayer cultures of D16 cells and transfected cell lines were maintained in 95 % OptiMEM-I, 5 % fetal bovine serum. The original AtT-20 cells were cultured in 85 % OptiMEM-I, 10 % equine serum, 5 % fetal bovine serum ; medium for routine culture of the transfected cells contained 200 μg/ml G418. All cell lines were incubated in humidified 95 % air, 5 % CO_2 at 37 ºC. The AtT-20/D16 cells were generously provided by Dr. Lee Limbird, Department of Pharmacology, Vanderbilt University, and the original AtT-20 cells were obtained from the American Type Culture Collection (CCL89).

The original AtT-20 cells were transfected using the LipofectAMINE reagent, according to the manufacturer's instructions. Briefly, 20 x 10^6 cells were transfected with 15 μg of the GAP-43 expression plasmid and 60 μl lipofectAMINE. After 6 h of incubation at 37 ºC, the medium was removed

and replaced by normal culture medium. After 72 h cells were split into selective medium containing 400 μg/ml G418. Clones were then isolated by limiting dilution and expanded in culture.

Intracellular Ca^{2+} measurements. Cells were spun and resuspended at 10^7 cell/ml in Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, 5.6 mM glucose, pH 7.4). Fura-2/acetoxy-methyl ester (fura2/AM, Molecular Probes) was added at a final concentration of 1 μM and loading was done for 30 min in the dark at room temperature. Unincorporated dye was removed by washing the cells once with KRH. Cells were resuspended at 0.5 x 10^6 cells/ml in KRH prewarmed at 37 ºC and transferred in fluorimeter cuvette and incubated at 37 ºC for 30 min, to allow complete de-esterification. Fura-2 fluorescence was measured using a LS50 luminescence spectrometer (Perkin-Elmer) and Ca^{2+} concentrations were calculated as described by Grynkiewicz *et al.* (47), using the Intracellular Biochemistry software package (Perkin-Elmer).

RNase protection analysis. Total cellular RNA was extracted using the AGPC method (48). Radiolabeled complimentary RNA probes were generated *in vitro* with [α-^{32}P]-UTP (Du Pont NEN) as label. Five μg of total RNA from each sample were ethanol-precipitated and re-dissolved in 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80 % formamide with 10^5 cpm of labeled RNA probe. The RNAs were denatured by heating to 85 ºC for 5 min, then hybridized at 48 ºC overnight. RNase protection analysis was performed by standard methods (49) using digestion with RNase T1 (Life Technologies, Inc.) for 1 h at 30 ºC. The resulting RNA fragments were resolved by electrophoresis in a 6 % acrylamide, 8 M urea sequencing gel, which was dried

and exposed to X-ray film (X-OMAT AR, Eastman Kodak Co.), with an intensifying screen at –70 ºC.

Immunoblot analysis. Total cellular proteins were extracted with RIPA buffer (10 mM TRIS, pH 7.2, 150 mM NaCl, 1 % deoxycholate, 1 % Triton X-100, 0.1 % SDS) containing 2 μg/ml aprotinin and quantified by the method of Bradford (50) using bovine serum albumin as a standard. Subcellular fractionation was performed by lysing cells in 20 mM Tris pH 7.4, 2 mM EDTA, 1 mM EGTA and separating the particulate and soluble fractions by centrifugation at 100 000 x *g*. Proteins were separated by SDS-polyacrylamide gel electrophoresis on SDS-10% polyacrylamide minigels (Hoefer Scientific Instruments) and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). Protein blots were blocked with 5 % non-fat milk in phosphate-buffered saline (PBS) and incubated with either an anti-rat GAP-43 polyclonal antibody (30) or an anti-GAP-43 monoclonal antibody (clone GAP-7B10, Sigma,) followed by a peroxidase-conjugated secondary antibody (Sigma,). Bound antibodies were detected by enhanced chemiluminescence (Du Pont NEN) and exposure to X-ray film.

Secretion studies. Cells were plated in 6-well cluster dishes at an initial density of 2.5 x 10^5 cells/well and were used for experiments 6 days later. For incubations with CRF, the medium was replaced by prewarmed OptiMEM-I containing BSA (2.5 mg/ml) and protease inhibitors (0.1 mg/ml trypsin inhibitor, 2 μg/ml aprotinin). After 30 min the medium was removed and replaced by fresh medium without (basal) or with 100 nM CRF (Sigma) and the cells were incubated an additional 30 min at 37 ºC. For K^+ stimulation, the cells were equilibrated for 15 min in KRH. The medium was then removed and the cells were incubated for 5 min either in KRH buffer (basal), or in KRH

buffer containing 56 mM KCl (in which the NaCl concentration was decreased to maintain iso-osmolarity). After the incubation with either CRF or elevated potassium the medium was collected, centrifuged 3 min at 1 700 x *g* to remove dislodged cells and debris and phenylmethylsulfonylfluoride added to a final concentration of 2 mM. The cells were collected in PBS, centrifuged and protein was extracted from the cell pellets with RIPA buffer containing protease inhibitors. Secreted and cellular hormones were measured by radioimmunoassay (RIA). Net secretion (CRF or K^+-stimulated minus basal) was expressed as the percent of total cellular stores of β–endorphin released during the incubation period.

Radioimmunoassay. β–endorphin immunoassays were performed as described previously (51), using an antiserum which is specific for β–endorphin residues 15-26. Synthetic acetyl-β–endorphin 1-27 was used as tracer and standard, and a 12-point standard curve was assayed with each group of samples. The unpaired 't' test was used to determine the statistical significance of the results.

RESULTS

Potassium-stimulated β–endorphin secretion is correlated with GAP-43 expression

Levels of GAP-43 in the original AtT-20 and D16 cell lines were determined by immunoblot analysis. As shown in figure 1, GAP-43 is undetectable in the original AtT-20 cells, but is expressed at high levels in the D16 cells. As previous investigations had suggested that GAP-43 might be involved in exocytosis (33, 34, 39), we analyzed evoked hormone secretion in these two cell lines.

Initial experiments with cultures of D16 cells demonstrated that 56 mM K^+- and 100 nM CRF-evoked β–endorphin secretion were linear for at least 5 and 30 minutes respectively (data not shown). Net CRF-stimulated secretion (stimulated minus basal) from the D16 cells averaged 6.9 % of the total cellular stores in 30 minutes and produced an identical secretory response in the original AtT-20 (figure 2A). All of the essential components of the secretory machinery are thus present and functional in both cell lines. In contrast to the results with CRF, potassium depolarization resulted in a marked stimulation of β–endorphin secretion from the D16 cells (17.7 % of total cellular stores in 5 minutes), but produced a dramatically lower amount of secretion from the original AtT-20 (net 1.6 % of total cellular stores ; figure 2B). We have verified by spectrofluorimetric analysis with the dye Fura2-AM that depolarization-mediated calcium influx is similar in these two cell lines (figure 3). These results prompted us to ask whether expression of GAP-43 in the original AtT-20 cells would restore potassium-evoked secretion.

Transfection of GAP-43 in the original AtT-20

The original AtT-20 cells were transfected with a plasmid in which expression of the rat GAP-43 cDNA was driven by the RSV promoter and permanently transfected cells were selected with G418. RNase protection analysis with a radiolabeled antisense RNA GAP-43 probe demonstrates that 5 cell lines, designated AtT-20:rGAP-43 #1, AtT-20:rGAP-43 G8D, AtT-20:rGAP-43 G4G, AtT-20:rGAP-43 K3F and AtT-20:rGAP-43 H5E, transcribe GAP-43 RNA from both the transfected GAP-43 cDNA and the endogenous gene (figure 4A, lanes 8-12). Four other G418-resistant cell lines that were obtained by transfecting the backbone plasmid pRc/RSV into AtT-20 cells and were designated as AtT-20:pRc/RSV BB1, AtT-20:pRc/RSV CC1, AtT-20:pRc/RSV DD1 and AtT-20:pRc/RSV DD2 (lanes 4-7), express only the endogenous GAP-43 mRNA, which is also readily detectable in the parental AtT-20 (lane 3) and D16 cell lines (lane 2).

The expression of GAP-43 protein in these cell lines was then analyzed by immunoblot (figure 4B). As demonstrated in figure 1, the D16 cells (lane 1) produce high amounts of GAP-43, but the protein is undetectable in the original AtT-20 (lane 2). The AtT-20:rGAP-43 #1, G8D, G4G, K3F and H5E cell lines, which transcribe GAP-43 RNA from the transfected cDNA, also produce significant amount of GAP-43 protein (lanes 7-11). In contrast, this polypeptide is undetectable in the control cell lines AtT-20:pRc/RSV BB1, CC1, DD1 and DD2, which express only the endogenous GAP-43 mRNA (lane 3-6).

In neurons, GAP-43 is mostly associated with the plasma membrane (7, 8, 10, 13, 52, 53) and when the GAP-43 cDNA is transfected into non-neuronal cells the protein shows a similar subcellular distribution (54-57). Subcellular fractionation indicated that GAP-43 localizes to the particulate fraction in the transfected AtT-20 and the D16 cell lines as well (data not shown).

Expression of GAP-43 in the original AtT-20 cells restores potassium-evoked secretion

The transfected AtT-20 cell lines were stimulated for 5 min with 56 mM KCl and secretion of β–endorphin was measured as described. These studies revealed that β–endorphin secretion in the GAP-43-expressing AtT-20:rGAP-43 cell lines was markedly stimulated (on the average 7.5 % of total cellular stores in 5 min). In contrast, secretion in the control AtT-20:pRc/RSV cell lines (2.4 % of total cellular stores in 5 min) was not significantly different from that in the parental AtT-20 cell line (Table 1). Potassium-evoked Ca^{2+} influx in the transfected cell lines (measured by spectrofluorimetric analysis using the dye Fura2-AM) was similar to that in the parental AtT-20 and D16 cells (figure 5). CRF-evoked hormone secretion in the transfected cells expressing GAP-43 was not significantly increased (data not shown).

GAP-43 induces morphological changes in AtT-20 cells

As GAP-43 has been shown to induce process outgrowth when transfected into non-neuronal cells (54, 58-62), we investigated if transfection of GAP-43 would also cause morphological changes in AtT-20 cells. The D16 cells grow as a monolayer, flatten, and extend processes when plated at low density (figure 6A). In contrast, the original AtT-20 cells normally grow in suspension but will attach to laminin-coated culture substrates while retaining a rounded morphology (figure 6B). The transfected cell lines also grow in suspension under routine culture conditions. However, when seeded on laminin-coated plates, about 30 % of the transfected cells expressing GAP-43 flattened and extended processes (figure 6C). These morphological changes were not observed for the control AtT-20:pRc/RSV CC1 cell line cultured in the same conditions (figure 6D).

DISCUSSION

We report here that transfection of GAP-43 into mouse anterior pituitary AtT-20 cells dramatically augments depolarization-mediated hormone secretion without a change in calcium influx. Additionally, induced expression of GAP-43 results in morphological alterations that include process outgrowth from the cells

GAP-43 promotes depolarization-mediated hormone secretion in AtT-20 cells

Expression of GAP-43 is not detectable in either the anterior lobe cells of the pituitary (63) or in the original AtT-20 cell line, which was derived from a mouse anterior pituitary tumor. In marked contrast, GAP-43 expression is robust in the AtT-20/D16 cell line that was subcloned from the original AtT-20. Our initial studies indicated that the secretory response to elevated extracellular potassium was well-correlated with the expression of GAP-43 in the two cell lines. The possibility that this differential response to membrane depolarization might be due to some difference between these cells other than GAP-43 expression is ruled out by the demonstration that transfection of GAP-43 into the original AtT-20 cells restores potassium-evoked secretion.

GAP-43 augments potassium depolarization-mediated hormone release without a significant effect on CRF-induced secretion. This differential effect may be due to the fact that these two secretagogues appear to act via different biochemical mechanisms, which have been characterized extensively in the AtT-20/D16 cells. CRF, the normal secretagogue for anterior pituitary corticotrophs, binds to cell-surface receptors coupled to adenylate cyclase through G_S. The ensuing increase in cellular cyclic AMP levels activates cAMP-dependent protein kinase which phosphorylates calcium channels (64).

This effect occurs within minutes after the addition of CRF to the cells. Potassium depolarization produces a much larger and more rapid calcium mobilization through voltage-gated channels (65), without a change in intracellular cAMP. GAP-43 may serve to facilitate transmission of the biochemical signal that is initiated by the rapid transient depolarization-induced influx of calcium.

GAP-43 has been characterized previously as a calmodulin binding protein with the unusual property of binding calmodulin under low calcium conditions and releasing it in response to elevations in calcium (16-18, 66). This property has led to the hypothesis that GAP-43 may serve to sequester calmodulin at the inner face of the plasma membrane to permit the rapid activation of calmodulin-dependent processes (66), including CaM kinase II, which has been implicated in neurotransmitter release (67, 68). The fact that the AtT-20 cells that do not express GAP-43 are nevertheless capable of calcium-dependent secretion of hormone in response to CRF suggests that the action of GAP-43 in the secretory process is likely to be indirect.

Our results are consistent with those of Gispen and colleagues, who have demonstrated that introduction of GAP-43 antibodies into permeabilized synaptosomes inhibits calcium-dependent GAP-43 phosphorylation and neurotransmitter release (34, 35, 37, 38). Similarly, Ivins *et al.* (39) showed that antisense RNA-mediated inhibition of GAP-43 expression in PC12 pheochromocytoma cells significantly diminishes depolarization-mediated catecholamine secretion. Possible mechanisms for the action of GAP-43 in secretion have been addressed in additional studies with synaptosomes using antibodies directed specifically against the amino-terminus of GAP-43, which have provided further evidence for a role of calmodulin in this process (38). Furthermore, introduction of GAP-43 peptides into permeabilized chromaffin

cells has been shown to modulate Ca^{2+}-regulated exocytosis via interactions with GTP-binding proteins (69). In contrast to these previous studies, most of which have relied upon the inhibition of GAP-43 function, our investigations have demonstrated a robust and readily quantifiable positive effect that results from the stable expression of GAP-43 in a well-characterized cell line. This suggests that AtT-20 cells will be a useful model system for future studies of the precise molecular mechanisms of GAP-43 action and may help to resolve some of the unresolved issues that remain from previous investigations (35).

GAP-43 produces morphological changes in AtT-20 cells

Forced expression of GAP-43 also caused the original AtT-20 cells to flatten and extend processes on laminin-coated culture substrata. These processes are clearly present for several days in culture, and are thus significantly more stable than the transient processes induced by GAP-43 in COS and CHO cells (54, 61, 62). The stability of this response in our studies suggests again that AtT-20 cells will be a useful model system for future investigations of the mechanism of GAP-43 action.

The morphological alterations noted here may relate to the postulated role of GAP-43 in axonal growth and regeneration, in which context this polypeptide was first identified (70, 71). A multitude of subsequent investigations has sought convincing evidence for a role of GAP-43 in process extension (reviewed in 10, 52, 72) ; the results of these studies have been somewhat equivocal. For example, although the expression of GAP-43 is highly correlated with axonal growth in a variety of experimental systems (7, 52, 73-80), the protein is absent from the dendrites of hippocampal pyramidal neurons that still extend prominently in culture (81). Furthermore, a line of

PC12 pheochromocytoma cells that lacks GAP-43 can elongate long branching processes in response to Nerve Growth Factor (82). Phosphatidylcholine-mediated introduction of GAP-43 antibodies into NB2a/d1 neuroblastoma cells in culture does however prevent the initial phase of neurite outgrowth in response to cyclic AMP (83).

This variability in the requirement for GAP-43 may result from the fact that the precise function of this protein is dependent upon the cell type in which it is expressed. Alternatively, the role of GAP-43 in axonal growth may be indirect, as suggested by recent studies on the effects of disruption of the GAP-43 gene in the mouse embryo (84). The effects of GAP-43 on cellular morphology noted in the current study represent a positive effect of the induced expression of the protein, and again indicate the utility of AtT-20 cells for studies of the mechanism of action of this protein, which we are now pursuing.

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Figure 1

Immunoblot analysis reveals that GAP-43 is expressed at high levels in the D16 cells but is undetectable in the original AtT-20 cells. Proteins extracted from the original AtT-20 (odd numbered lanes) and D16 (even numbered lanes) cell lines were resolved by SDS/polyacrylamide gel electrophoresis and immunoblot analysis was performed as described under "Experimental Procedures", using a polyclonal anti-rat GAP-43 antibody. Lanes 1 and 2 contain 20 μg, lanes 3 and 4 : 10 μg and lanes 5 and 6 : 5μg of protein.

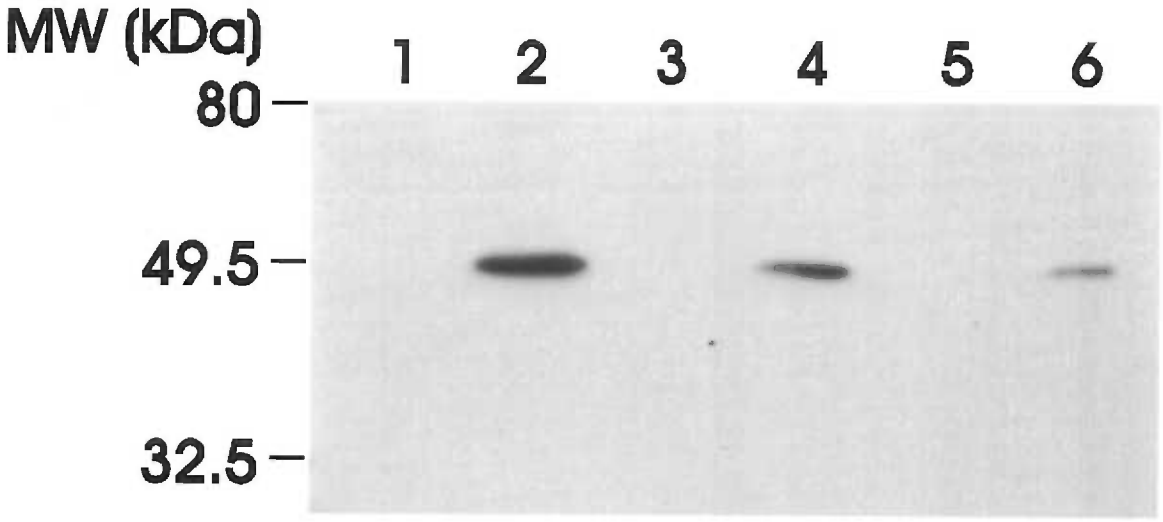
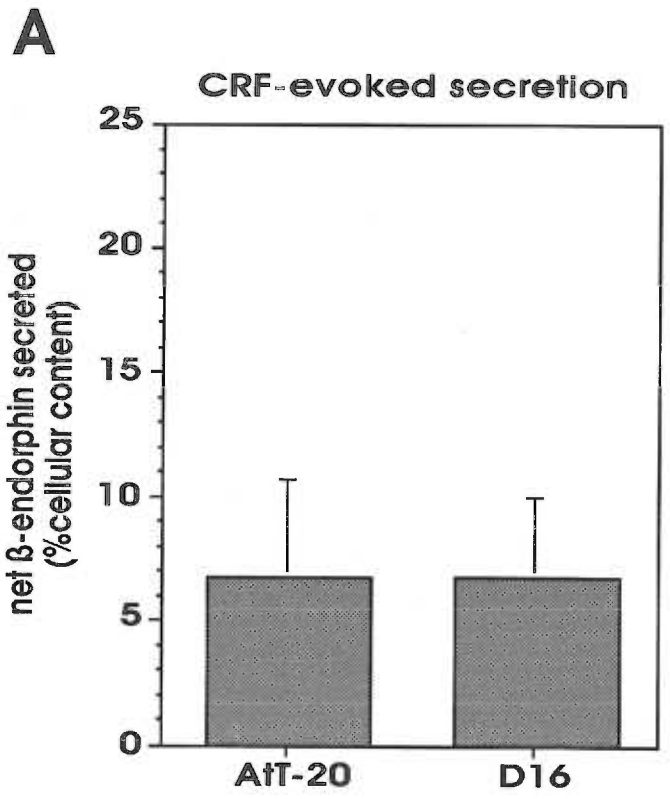

MW (kDa)
1
2
3
4
5
6
80
49.5
32.5

Figure 2

CRF- and potassium-evoked secretion of ß-endorphin in cultures of the original AtT-20 and the D16 cells. Secretion experiments and quantitation of β-endorphin by radioimmunoassay were performed as described in Experimental Procedures. Panel A, net CRF-evoked (CRF-evoked minus basal) secretion of ß-endorphin from the two cell lines. Cultures were incubated for 30 min at 37 ºC in either control medium (basal) or in medium containing 100 nM CRF. Basal release was on the average 4.6 % ± 2.2 (D16) and 13.5 % ± 6.0 (AtT-20). Panel B, net potassium-evoked (potassium-evoked minus basal) secretion of β-endorphin from the two cell lines. Cultures were incubated for 5 min at 37 ºC in either KRH buffer (basal) or in buffer containing 56 mM KCl. Basal release was on the average 3.7 % ± 1.7 (D16) and 6.9 % ± 4.1 (AtT-20). The values shown here represent the mean of 3 separate determinations, each carried out in triplicate. Error bars indicate the standard deviation of the mean. *$p<0.05$ significantly different from the net β-endorphin release from the original AtT-20 cells.


A
CRF-evoked secretion
net ß-endorphin secreted
(%cellular content)
25
20
15
10
5
0
AtT-20
D16

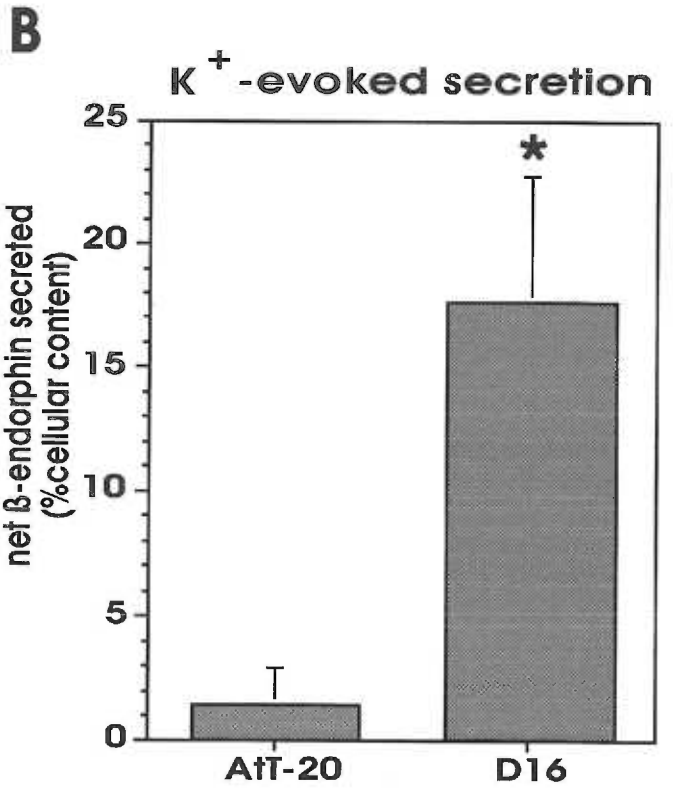
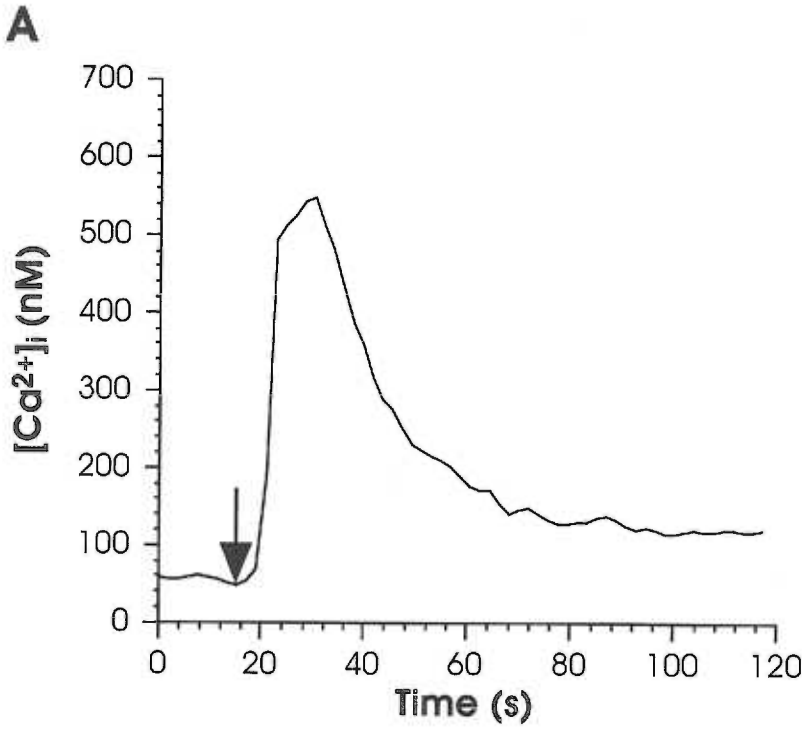

B
K+-evoked secretion
net ß-endorphin secreted
(%cellular content)
25
20
15
10
5
0
*
AtT-20
D16

Figure 3

K^+-evoked influx of calcium in AtT-20 cells. Cells were loaded with the fluorescent dye fura2/AM. At the time indicated by the arrow, KCl was added to the cells to a final concentration of 56 mM and intracellular calcium was measured as described under "Experimental Procedures". Panel A, intracellular calcium concentration in D16 cells. Panel B, intracellular calcium concentration in the original AtT-20 cells. The graphs shown are representative of at least 3 independent determinations.


A
700
600
500
400
300
200
100
0
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (s)

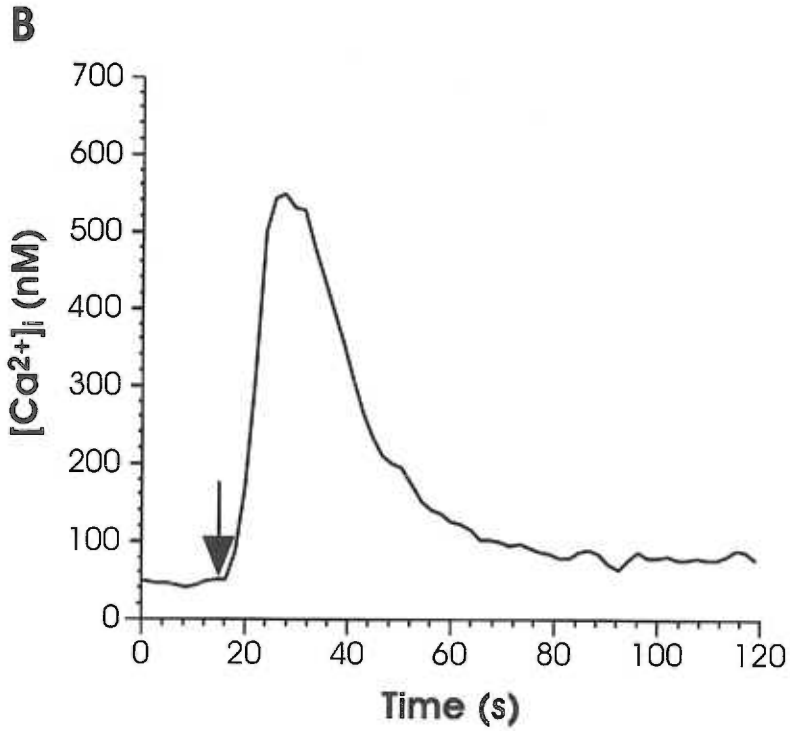

B
700
600
500
400
300
200
100
0
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (s)

Figure 4

Expression of GAP-43 in the transfected AtT-20 cells. Original AtT-20 were transfected with the expression plasmid for rat GAP-43 and stably transformed cell lines were selected as described under "Experimental Procedures". Panel A, RNase protection analysis of GAP-43 RNA expression in the original AtT-20 cells, D16 cells and several transfected cells lines. Lane 1, intact GAP-43 (300 nt) and β-actin (245 nt) riboprobes. The GAP-43 riboprobe includes some pGEM 3Zf sequences so the protected fragments are shorter. This riboprobe will protect two fragments : the larger (approximately 270 nt, exogenous) results from hybridization with the mRNA transcribed from the transfected rat GAP-43 cDNA, and the smaller fragment (approximately 240 nt, endogenous) results from hybridization with the endogenous GAP-43 transcript ; lane 2, D16 ; lane 3, original AtT-20 ; lane 4, AtT-20:pRc/RSV BB1 ; lane 5, AtT-20:pRc/RSV CC1 ; lane 6, AtT-20:pRc/RSV DD1 ; lane 7, AtT-20:pRc/RSV DD2 ; lane 8, AtT-20:rGAP-43 #1 ; lane 9, AtT-20:rGAP-43 G8D ; lane 10, AtT-20:rGAP-43 G4G ; lane 11, AtT-20:rGAP-43 K3F ; lane 12, AtT-20:rGAP-43 H5E ; lane 13, control yeast tRNA. Panel B, Immunoblot analysis of GAP-43 expression in the original AtT-20 cells, D16 cells and the transfected AtT-20 cell lines. 20 μg of protein were resolved on a 10 % SDS/polyacrylamide gel and immunoblot analysis was performed as described previously with a monoclonal anti-GAP-43 antibody. Lane 1, D16 ; lane 2, original AtT-20 ; lane 3, AtT-20:pRc/RSV BB1 ; lane 4, AtT-20:pRc/RSV CC1 ; lane 5, AtT-20:pRc/RSV DD1 ; lane 6, AtT-20:pRc/RSV DD2 ; lane 7, AtT-20:rGAP-43 #1 ; lane 8, AtT-20:rGAP-43 G8D ; lane 9, AtT-20:rGAP-43 G4G ; lane 10, AtT-20:rGAP-43 K3F ; lane 11, AtT-20:rGAP-43 H5E.

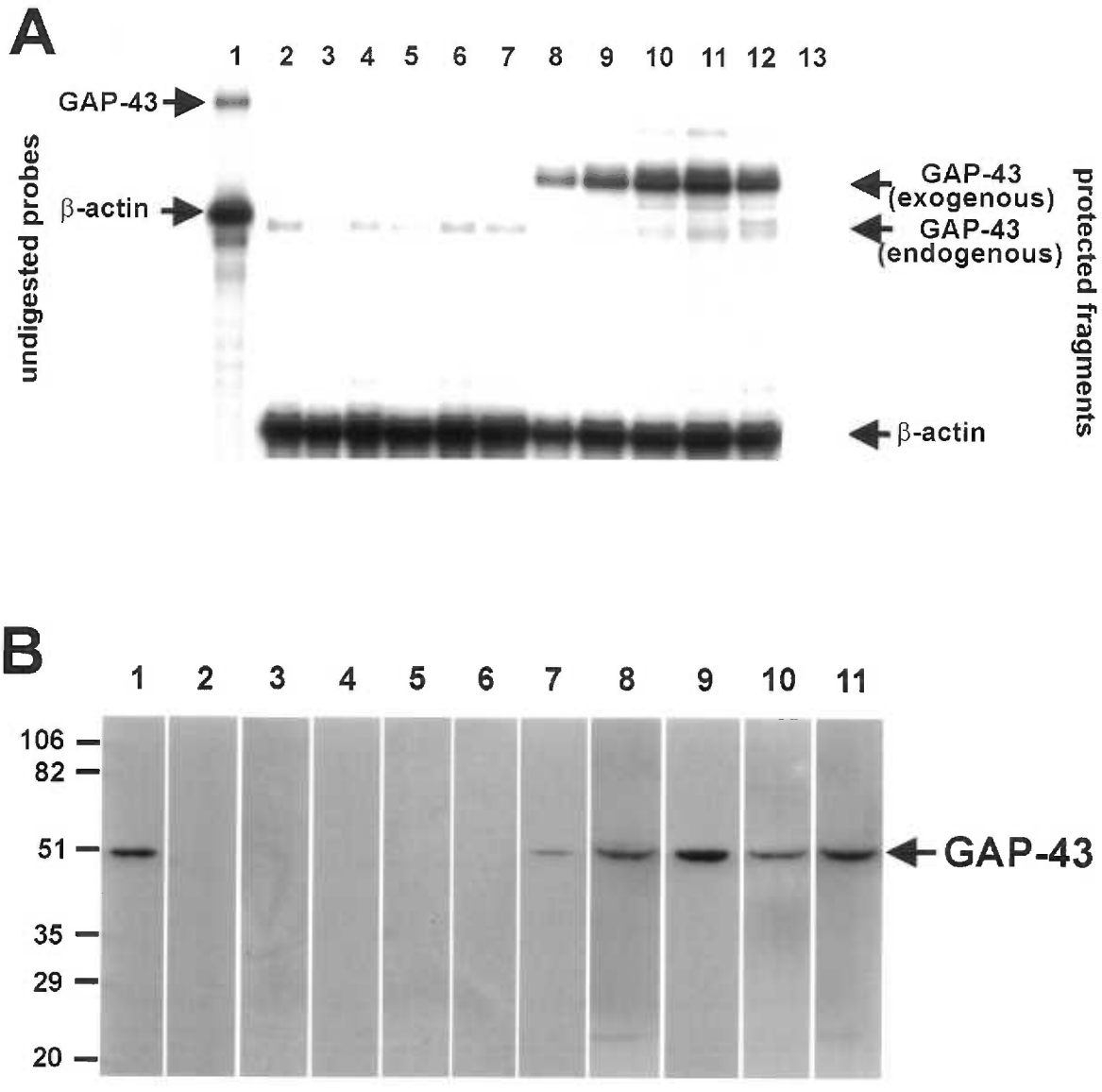

A
1 2 3 4 5 6 7 8 9 10 11 12 13
GAP-43
β-actin
undigested probes
GAP-43 (exogenous)
GAP-43 (endogenous)
β-actin
protected fragments
B
1 2 3 4 5 6 7 8 9 10 11
106
82
51
35
29
20
GAP-43

TABLE I

Net potassium-stimulated secretion of ß-endorphin from transfected AtT-20 clones

Stimulation of secretion in the cell lines was determined by measuring basal and K^+-evoked release of ß-endorphin as described in experimental procedures. Basal release was on the average 1.4 % ± 0.3 for AtT-20:pRc/RSV clones and 4.4 % ± 4.2 for AtT-20:ratGAP-43 clones and were statistically not significantly different between these two groups.

Plasmid	Clone number	net K^+-evoked secretion[a]
pRc/RSV vector	BB1	2.3 ± 0.1
	CC1	2.9 ± 1.2
	DD1	2.3 ± 1.2
	DD2	2.0 ± 0.4
	average	2.4 ±0.4
pRc/RSV-ratGAP-43	#1	11.1 ± 5.1[b]
	G8D	8.9 ± 2.6[b]
	G4G	5.2 ± 0.5[b]
	H5E	6.3 ± 1.2[b]
	K3F	6.1 ± 2.4[b]
	average	7.5 ± 2.4 [c]

a) Net secretion (K^+-evoked minus basal) is expresssed as the percent of total cellular stores of β-endorphin ; values are mean ± S. D. of at least 3 determinations done in triplicate.

b) significantly different from each AtT-20:pRc/RSV clone, at least $p < 0.05$.

c) significantly different from the average of the AtT-20:pRc/RSV clones, $p < 0.005$.

Figure 5

K^+-evoked influx of calcium in transfected AtT-20 cells. Cells were loaded with the fluorescent dye fura2/AM. At the time indicated by the arrow, KCl was added to the cells to a final concentration of 56 mM and intracellular calcium was measured as described under "Experimental Procedures". Panel A, intracellular calcium concentration in AtT-20:pRc/RSV CC1 cells. Panel B, intracellular calcium concentration in AtT-20:rGAP-43 #1 cells. The graphs shown are representative of K^+-evoked Ca^{2+} influx in AtT-20:pRc/RSV and AtT-20:rGAP-43 cell lines. At least 3 independent determinations were performed for each cell line.

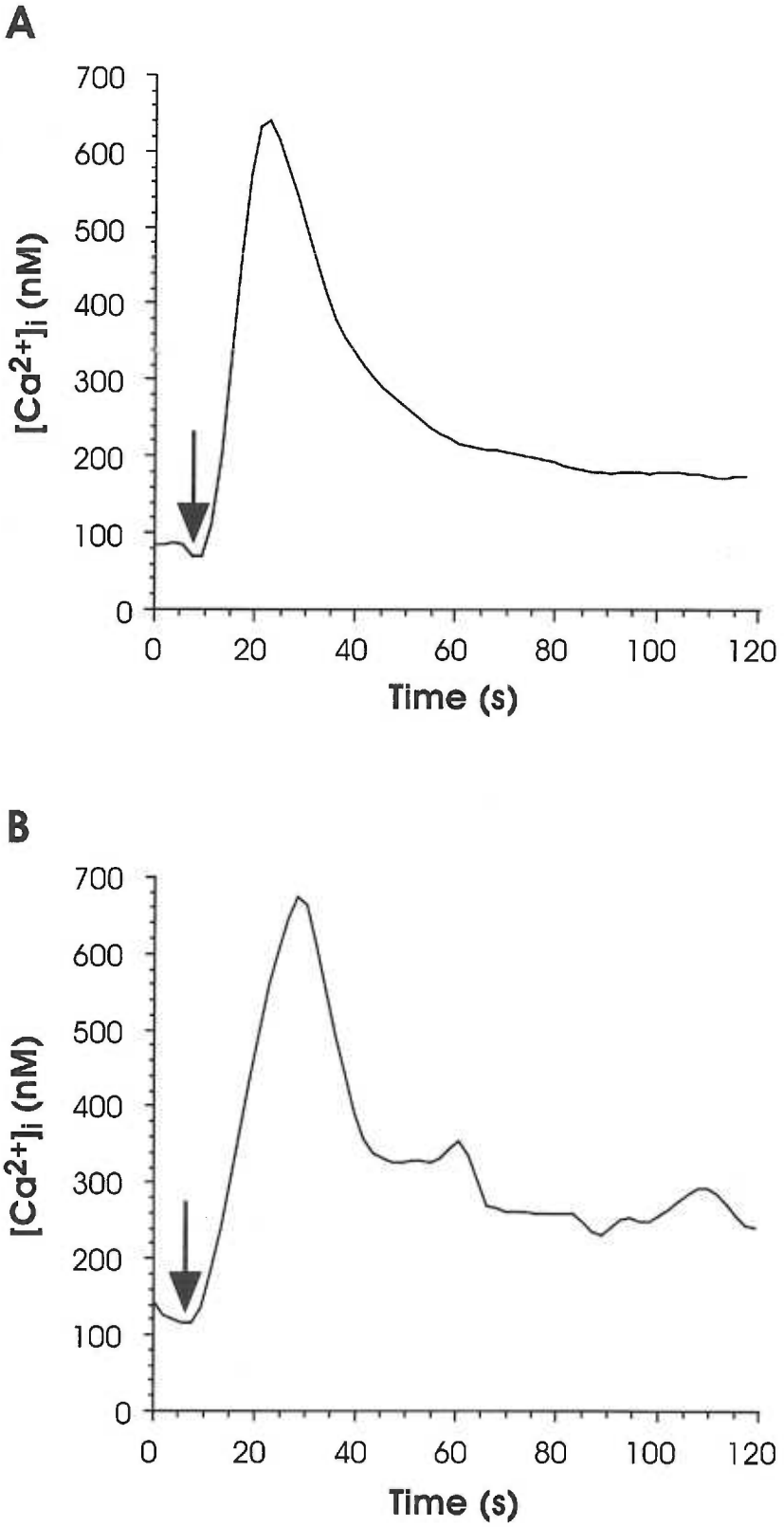
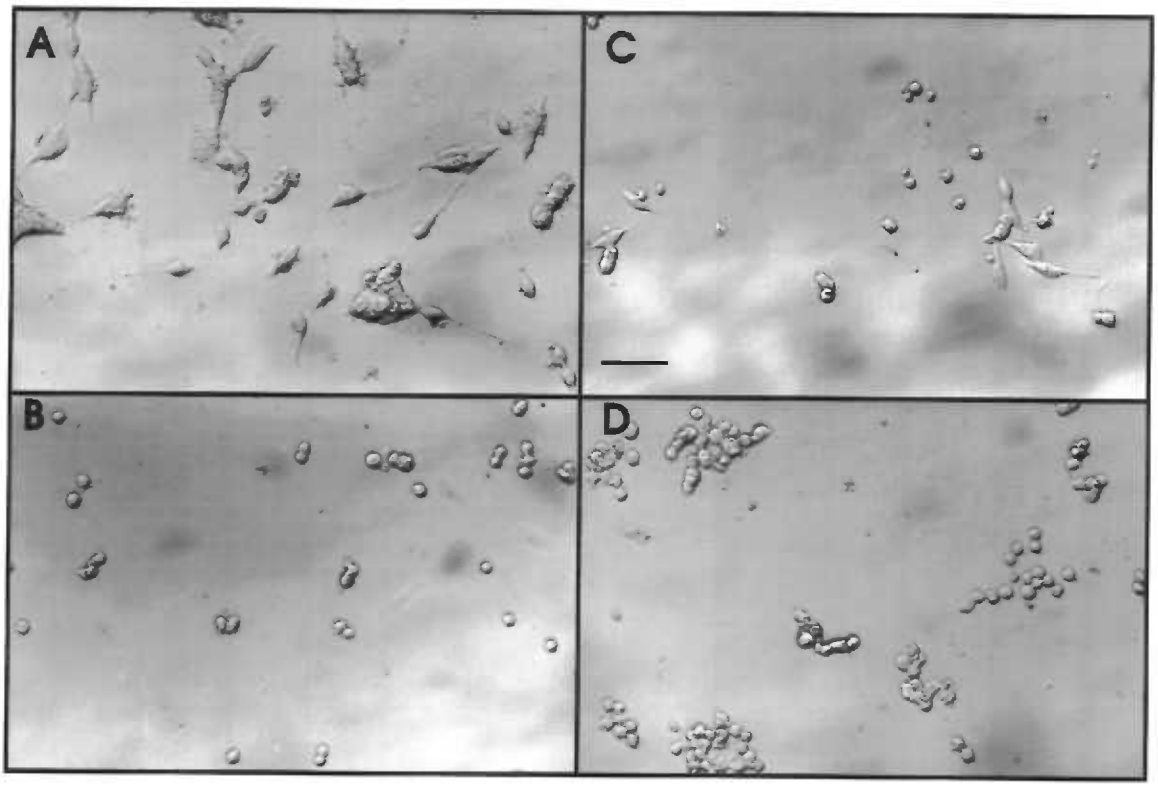

A
700
600
500
400
300
200
100
0
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (s)
B
700
600
500
400
300
200
100
0
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (s)

Figure 6

Transfection and expression of GAP-43 into AtT-20 cells induces morphological changes. D16 cells (panel A), original AtT-20 cells (panel B), AtT-20:rGAP-43 #1 cells (panel C) or AtT-20:pRc/RSV CC1 control cells (panel D) were plated at an initial density of 10^5 cells/plate on 35-mm plates coated with laminin (10 μg/plate) and cultured for 3 days before fixation. Scale bar, 50 μm.


A
C
B
D

MANUSCRIPT #2

Analysis of the role of calmodulin binding and sequestration in GAP-43 function

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Running title : GAP-43 and calmodulin binding

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The abbreviations used are :

BSA : bovine serum albumin ; DMSO : dimethylsulfoxide ; DSP : dithiobis (succinimidyl propionate) ; KRH : Krebs-Ringer-Hepes ; PVDF : polyvinylidene difluoride ; RSV : Rous sarcoma virus

SUMMARY

We demonstrated previously that forced expression of the neuronal Growth-Associated Protein (GAP)-43 in mouse anterior pituitary AtT-20 cells enhances depolarization-mediated secretion and alters cellular morphology. Here we analyze the role of calmodulin binding by GAP-43 in these responses. In cells expressing wild-type GAP-43 a complex with calmodulin that is sensitive to intracellular calcium and phosphorylation is localized to the plasma membrane. Transfection of several mutant forms of GAP-43 shows that the effects of this protein on secretion are dependent on both calmodulin binding and association with the plasma membrane. In contrast, the morphological changes depend only on membrane association. Thus the multitude of effects of GAP-43 noted in previous studies may result from divergent properties of this protein.

INTRODUCTION

The growth associated protein (GAP)-43 (neuromodulin, B-50, F1) is a membrane-bound phosphoprotein expressed at a high level during neuronal development and regeneration (reviewed in Benowitz and Routtenberg, 1987; Skene, 1989). GAP-43 is a rapidly transported axonal protein (Allsopp and Moss, 1989 ; Basi *et al.*, 1987 ; Biffo *et al.*, 1990 ; de La Monte *et al.*, 1989 ; Jacobson *et al.*, 1986 ; Kalil and Skene, 1986 ; Moya *et al.*, 1987 ; Skene *et al.*, 1986 ; Skene and Willard, 1981) that is concentrated in the growth cone of elongating axons (Allsopp and Moss, 1989 ; de Graan *et al.*, 1985 ; Gorgels *et al.*, 1989 ; Goslin *et al.*, 1988 ; Goslin *et al.*, 1990 ; Meiri, 1986 ; Skene *et al.*, 1986 ; Van Lookeren Campagne *et al.*, 1990). Additionally, over-expression of GAP-43 in the nervous system of transgenic mice causes spontaneous nerve sprouting at the neuromuscular junction and potentiates lesion-induced nerve sprouting and terminal arborization during re-innervation (Aigner *et al.*, 1995). Taken together, these results suggest that GAP-43 plays an important role in axon elongation. Further support for this notion has been derived from studies of several cell culture model systems (Aigner and Caroni, 1993 ; Aigner and Caroni, 1995 ; Burry *et al.*, 1991 ; Kumagai *et al.*, 1992; Kumagai-Tohda *et al.*, 1993 ; Shea *et al.*, 1991 ; Widmer and Caroni, 1993 Strittmatter *et al.*, 1994b ; Yankner *et al.*, 1990 ; Zuber *et al.*, 1989a).

However, a line of PC12 cells in which GAP-43 expression is nearly undetectable is nevertheless capable of robust neurite elongation in response to Nerve Growth Factor (Baetge and Hammang, 1991). Additionally, cultured neurons derived from mice in which the GAP-43 gene has been disrupted by gene targeting extend axons to the same extent as cells that express this

protein. Further analysis of the GAP-43(-) embryos revealed that retinal axons appear incapable of crossing the midline decision point in the optic chiasm, implying that their growth cones fail to respond to environmental guidance cues (Strittmatter *et al.*, 1995). These results suggest that GAP-43 is perhaps not essential for axon elongation, but rather might function as a mediator of signal transduction pathways in the growth cone that serve to modulate the rate, extent and trajectory of axonal growth.

In the adult nervous system GAP-43 expression persists in pre-synaptic terminals in those regions where the synaptic modifications associated with learning and memory are thought to occur (Benowitz *et al.*, 1988 ; Benowitz *et al.*, 1989 ; Gispen *et al.*, 1985 ; Masliah *et al.*, 1991 ; Neve *et al.*, 1988 ; Neve *et al.*, 1987). Additionally, the correlation of PKC-mediated GAP-43 phosphorylation with long term potentiation of synaptic transmission in the hippocampus (Chan *et al.*, 1986 ; Gianotti *et al.*, 1992 ; Lovinger *et al.*, 1985 ; Routtenberg *et al.*, 1985) and neurotransmitter release *in vitro* (Dekker *et al.*, 1989) suggest a role for GAP-43 in neuronal plasticity, possibly via modulation of neurotransmitter release. Support for this notion is derived from the demonstration that antibodies to GAP-43 that prevent its phosphorylation inhibit evoked neurotransmitter release in permeabilized synaptosomes (Dekker *et al.*, 1989 ; Hens *et al.*, 1995 ; Hens *et al.*, 1993). Furthermore, the levels of GAP-43 mRNA expression and neurotransmitter release are well-correlated in NG108-15 cells (Kumagai-Tohda *et al.*, 1993). Finally, inhibition of GAP-43 expression in PC12 cells by antisense RNA markedly diminishes depolarization-mediated secretion of noradrenaline (Ivins *et al.*, 1993 ; Neve *et al.*, 1992).

GAP-43 appears to interact with several second messenger systems in the growth cone and nerve terminal. The N-terminal domain of GAP-43 has

been shown to stimulate GTP/GDP exchange on the a subunit of the GTP-binding proteins G_o or G_i (Strittmatter *et al.*, 1994a ; Strittmatter *et al.*, 1990 ; Strittmatter *et al.*, 1991). Injection of GAP-43 or GAP-43 N-terminal peptides in cultured DRG neurons or *Xenopus laevis* oocytes enhanced G-protein-mediated intracellular signaling (Igarashi *et al.*, 1993 ; Strittmatter *et al.*, 1993). In PC12 cells GAP-43 also appears to be modulate the function of L-type calcium channels, thereby affecting their electrical excitability (Baetge *et al.*, 1992). Additionally, as an inhibitor of the phosphatidylinositol 4-phosphate kinase (PIP) kinase, GAP-43 influences the metabolism of phosphoinositides and may act therefore as a feedback inhibitor of calcium mobilization and PKC activity (Jolles *et al.*, 1980). GAP-43 was also shown recently to be ADP-ribosylated (Coggins *et al.*, 1993), however the functional significance of this is unknown.

GAP-43 has also been shown to bind to calmodulin, with a higher affinity in the absence of Ca^{2+} than is its presence. Calmodulin binding is also disrupted by phosphorylation of GAP-43 by the Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Chapman *et al.*, 1991). These results have led to the hypothesis that GAP-43 serves to sequester calmodulin at the inner face of the plasma membrane in close proximity to calmodulin-dependent enzymes. Elevation of intracellular calcium would promote dissociation of calmodulin from GAP-43, allowing the rapid activation of these substrates (Apel and Storm, 1992 ; Estep *et al.*, 1990 ; Liu and Storm, 1990). The abundance of GAP-43 in the nerve terminal, together with its relative affinity for calmodulin suggest that GAP-43 might be a major neuronal calmodulin-binding protein. While studies have demonstrated that GAP-43 in synaptic plasma membranes binds exogenous radiolabeled calmodulin, the functional

significance of the GAP-43/calmodulin interaction in the intact cell remains to be explored.

We reported recently that forced expression of GAP-43 in mouse anterior pituitary AtT-20 cells enhanced K^+-evoked hormone secretion and induced changes in cellular morphology (Gamby *et al.*, 1996, manuscript#1). These results suggested that AtT-20 cells would be a useful experimental system to explore the molecular mechanism(s) of GAP-43 action. Here, we describe the results of studies designed to test the hypothesis that GAP-43 modulates K^+-evoked secretion from AtT-20 cells by sequestering CaM at the plasma membrane and releasing it upon elevation of intracellular Ca^{2+}.

RESULTS

Identification of a protein cross-linked to GAP-43. While GAP-43 has been shown to interact with CaM *in vitro* (Andreasen *et al.*, 1981 ; Andreasen *et al.*, 1983 ; Cimler *et al.*, 1985) and in purified synaptosomal plasma membranes (De Graan *et al.*, 1990), this interaction has yet to be demonstrated *in vivo.* Because the affinity of CaM for GAP-43 has been reported to be relatively low, we chose to use the approach of protein cross-linking to determine if CaM binds to GAP-43 in the intact cell. For these studies we used AtT-20/D16 (D16) cells, which express high levels of GAP-43 (Gamby *et al.*, 1996 ; manuscript #1), and the thiol-cleavable homobifunctional cross linker DSP, which due to its lipophilic nature penetrates into the cytoplasm of living cells (de Gunzburg *et al.*, 1989).

Cultures of AtT-20/D16 cells were treated with 1 mM DSP for 30 min, proteins were extracted and analyzed by Western blot with a GAP-43 monoclonal antibody. Two discrete immunoreactive proteins are apparent, migrating at approximately 50 kDa and 70 kDa (figure 1a, lane 4). The lower molecular weight corresponds to that reported for GAP-43 in a variety of studies (rviewed in Benowitz and Routtenberg, 1987), and is present in both control and cross-linked cells (figure 1a, lanes 2 and 4). The higher molecular weight species appears only after cross-linking, (figure 1a, lane 4) and is absent from membrane fractions from control cells (figure 1a, lane 2), or after reduction of cross-linked samples with b-mercaptoethanol (figure 1a, lane 3). To determine if the 70 kDa GAP-43-immunoreactive species results from the association of GAP-43 with CaM, we performed immunoblot analysis with an anti-CaM monoclonal antibody (figure 1b). In membrane fractions from control cells only a 19 kDa CaM immunoreactive band is detected (figure 1b,

lanes 1 and 2). In contrast, additional higher molecular weight species are detectable in the membrane fractions of cross-linked cells, with the most prominent migrating at approximately 70 kDa (figure 1b, lane 3). Quantitation by scanning densitometry indicates that as much as 70 % of the CaM in the particulate fraction is present in this 70 kDa complex. The higher molecular weight CaM-immunoreactive bands result from cross-linking since they are absent from reduced samples (figure 1b, lane 4). These results provide support for the notion that in the intact AtT-20 cell, GAP-43 and CaM are associated and form a 70 kDa complex.

To analyze this association further, the original AtT-20 cells, which lack detectable expression of GAP-43 (Gamby *et al.*, 1996, manuscript#1) and the D16 cells were cross-linked as described previously, and GAP-43 and CaM were detected in particulate and soluble cellular fractions by immunoblot analysis with anti-GAP-43 or anti-CaM antibodies respectively. GAP-43 is detectable only in the particulate fraction of D16 cells (figure 2, lane 1) and is absent from the soluble fraction of the D16 cells and the particulate fraction of the original AtT-20 cells (figure 2, lane 2-4). Both cell lines have similar levels of CaM, which is mostly a soluble protein (figure 2, lanes 6 and 8), but some CaM is also present in membrane fractions (figure 2, lanes 5 and 7). In the particulate fraction from cross-linked D16 cells, which express GAP-43, 70 % of CaM is present in a 70 kDa complex (figure 2, lane 5). This complex is not detected in the absence of GAP-43, either in the soluble fraction of D16 cells (figure 2, lane 6) or in the original AtT-20 cells (figure 2, lanes 7 and 8), suggesting that the 70 kDa complex results from the association of GAP-43 and CaM.

Finally, immunoprecipitation of solubilized membrane proteins from cross-linked D16 cells with a polyclonal anti-GAP-43 antibody, followed by

immunoblot analysis with a monoclonal anti-CaM antibody of the material eluted from the immune complex provides additional evidence that CaM is associated with GAP-43 in the intact AtT-20 cells (figure 3).

Intracellular calcium elevation or PKC phosphorylation of GAP-43 disrupt the association between GAP-43 and CaM. Extensive *in vitro* analysis of the GAP-43/CaM interaction has demonstrated that the affinity of GAP-43 for CaM is higher in the absence of Ca^{2+} than in the presence of 1 mM Ca^{2+} (Alexander *et al.*, 1987), leading to the suggestion that an elevation of intracellular calcium would promote dissociation of calmodulin from GAP-43 (Alexander *et al.*, 1987 ; Apel and Storm, 1992). To determine if this is in fact occurring in the intact cell we performed cross-linking and immunoblot experiments with D16 cells cultured in conditions that induce elevation of intracellular Ca^{2+}. D16 cells were treated with the Ca^{2+} ionophore A23187 (10 μM) for 5 min at 20 °C or with 0.1 % DMSO (control), then cross-linked with 1 mM DSP for 5 min at 20 °C and membrane proteins were analyzed by immunoblot analysis with anti-GAP-43 and anti-CaM antibodies (figure 4a). In the absence of the Ca^{2+} ionophore a prominent 70 kDa band corresponding to the GAP-43/CaM complex is detectable with both antibodies (figure 4a, lane 2 and 4). Treatment of the cells with the Ca^{2+} ionophore caused a marked reduction in the intensity of this 70 kDa band (approximately 90 % reduction, figure 4a, lanes 1 and 3). Membrane depolarization is an alternative method for inducing an elevation of intracellular Ca^{2+} in AtT-20 cells (Gamby *et al.*, 1996). Cultures of D16 cells were therefore also incubated for 5 min in KRH solution containing 56 mM KCl, prior to cross-linking with DSP, followed by analysis of membrane extracts by immunoblot analysis (figure 4b). The transient elevation of intracellular Ca^{2+} produced by depolarization also

caused a significant reduction of the complex between GAP-43 and CaM (figure 4b, lanes 2 and 4).

Phosphorylation of GAP-43 by the calcium- and phospholipid-dependent protein kinase (PKC) on ser^{41}, a residue in the CaM binding domain, has also been shown to interfere with the binding of CaM to GAP-43 (Chapman *et al.*, 1991). D16 cells were treated for 5 min with 1 μM TPA, an activator of PKC, prior to cross-linking with DSP, followed by analysis of membrane extracts by immunoblot analysis (figure 4c). Phosphorylation of GAP-43 by PKC also caused a significant 50 % reduction in the relative abundance of the GAP-43/CaM complex (figure 4c, lanes 2 and 4).

In primary cultures of hippocampal neurons the association of GAP-43 and CaM is also regulated by calcium and phosphorylation. In the mature nervous system, GAP-43 is expressed in nerve terminals of regions associated with learning and memory (Benowitz *et al.*, 1988 ; Benowitz *et al.*, 1989 ; Gispen *et al.*, 1985 ; Masliah *et al.*, 1991 ; Neve *et al.*, 1988 ; Neve *et al.*, 1987). We decided therefore to investigate GAP-43 binding to CaM in isolated hippocampal neurons. Cultures (8 days after initiation) were treated in the same conditions that have been shown to disrupt the complex between GAP-43 and CaM: 10 μM A23187, 1 μM TPA or 56 mM KCl for 5 min at 20 ºC or with 0.1 % DMSO or KRH alone (controls), and then cross-linking and immunoblot analysis were performed as described. In control cultures a prominent 70 kDa band corresponding to the GAP-43/CaM complex is detectable with anti-GAP-43 antibodies (figure 5a, lane 1 and 6) and with anti-CaM antibodies (figure 5b, lane 1 and 6). Treatment of the cultures with the Ca^{2+} ionophore A23187, TPA or elevated potassium caused a marked

reduction in the intensity of this 70 kDa band (approximately 85 % reduction, figure 5ab, lanes 2 -5), similar to the results obtained in the AtT-20/D16 cells.

Requirement of CaM for secretion. The above results verify that GAP-43 is associated with CaM in the intact cell, and that this interaction is regulated by intracellular Ca^{2+} concentration and phosphorylation. We therefore proceeded to determine if CaM is necessary for depolarization-evoked secretion in the AtT-20 cells. To block the function of CaM, we used 3 different cell permeable CaM antagonists : calmidazolium chloride (Silver, 1986), trifluoperazine dimaleate (Massom, 1990) and W7 (Itoh and Hidaka, 1984). D16 cells (Gamby *et al.*, 1996), were treated for 30 min with increasing concentrations of the CaM antagonist prior to stimulation with KRH containing 56 mM KCl (K^+-evoked release) or KRH (basal release). All three CaM antagonists inhibit K^+-evoked secretion of b-endorphin in a dose-dependent fashion (figure 6). The IC_{50} was 4 μM for calmidazolium chloride (figure 6a), 8.5 μM for trifluoperazine dimaleate (figure 6b) and 20 μM for W7 (figure 6c).

Transfection of AtT-20 cells with mutants of GAP-43. After verifying that CaM is necessary for K^+-evoked secretion in AtT-20 cells, we investigated whether GAP-43-mediated concentration of CaM at the plasma membrane is also required. In neurons, GAP-43 is mostly associated with the plasma membrane (Benowitz and Routtenberg, 1987 ; Gorgels *et al.*, 1989 ; Meiri and Gordon-Weeks, 1990 ; Meiri *et al.*, 1986 ; Skene, 1989 ; Skene *et al.*, 1986) and when the GAP-43 cDNA is transfected into non-neuronal cells the protein shows a similar subcellular distribution (Liu *et al.*, 1991 ; Liu *et al.*, 1993 ; Liu *et al.*, 1994 ; Zuber *et al.*, 1989a). The original AtT-20 cells, which lack GAP-43 (Gamby *et al.*, 1996, manuscript#1) were transfected with plasmids in which

expression of the [S41D]ratGAP-43 mutant cDNA or the [C3,4G]ratGAP-43 mutant cDNA were driven by the RSV promoter. Binding of CaM to GAP-43 has been shown to be abolished by mutation of ser 41, a residue in the CaM-binding domain, to aspartate (Chapman *et al.*, 1991). Mutation of the 2 cysteine residues cys 3 and cys 4 in the N-terminal domain of GAP-43 to glycine has been shown to prevent GAP-43 association with the plasma membrane (Chapman *et al.*, 1992 ; Zuber *et al.*, 1989b).

Permanently transfected cells were selected with G418, and five independent GAP-43-expressing cell lines were obtained for each transfection. Subcellular fractionation was performed and expression of GAP-43 protein in these cell lines was then analyzed by immunoblot (figure 7). The 5 AtT-20:[S41D]ratGAP-43 and the 5 AtT-20:[C3,4G]ratGAP-43 produce significant amount of GAP-43 protein. Most of [S41D]ratGAP-43 localizes to the particulate fraction in the transfected AtT-20 cells (figure 7a). However, [C3,4G]ratGAP-43 is present only in the soluble fraction of the cell lines transfected with this mutant (figure 7b).

Association of CaM with GAP-43 in mutants. Investigation of the binding of CaM to [S41D]GAP-43 by cross-linking and immunoblot analysis as described previously shows that although both GAP-43 and CaM are readily detectable in the cell lines expressing this mutant, the 70 kDa species representing the GAP-43/CaM complex is undetectable with either antibody, indicating that CaM cannot associate with [S41D]GAP-43. In contrast, the [C3,4G]GAP-43 mutant does bind to CaM, as demonstrated by the presence of a 70 kDa band, detected with both anti-GAP-43 and anti-CaM antibodies, in the soluble fraction of the cell lines expressing this mutant form of GAP-43 (figure 7b).

Effect of mutants of GAP-43 on secretion. The transfected AtT-20 cell lines were stimulated for 30 min with 100 nM CRF or for 5 min with 56 mM KCl and secretion of b-endorphin was measured as described. These studies revealed that CRF-evoked hormone secretion in the transfected cell lines expressing the mutant forms of GAP-43 (8 % of total cellular stores in 30 min, Table I) was similar to that in the parental AtT-20 and D16 cells (Gamby *et al.*, 1996, manuscript#1). All of the essential components of the secretory machinery are thus present and functional in all cell lines. However, K^+-evoked β-endorphin secretion in the AtT-20:[S41D]ratGAP-43 and in the AtT-20:[C3,4G]ratGAP-43 cell lines was low (on the average 2.6 % of total cellular stores in 5 min, Table I) and not significantly different from that in the parental AtT-20 cell line or AtT-20 cell lines obtained from transfection with the pRc/RSV vector alone (Gamby *et al.*, 1996, manuscript#1). Hence, the mutant forms of GAP-43 which cannot bind CaM or associate with the plasma membrane fail to enhance K^+-evoked release in the transfected cells.

In control experiments we have verified by spectrofluorimetric analysis with the dye Fura2-AM that depolarization-mediated calcium influx is not impaired in these cell lines (figure 9). We also have verified by immunoblot analysis with an anti-CaM antibody that the levels of CaM expression in the transfected cell lines are not significantly different from those in the control cell lines (data not shown).

Effects of mutant forms of GAP-43 on cellular morphology. As GAP-43 has been shown to induce process outgrowth when transfected into non-neuronal cells (Baetge *et al.*, 1992 ; Morton and Buss, 1992 ; Strittmatter *et al.*, 1994b ; Widmer and Caroni, 1993 ; Yankner *et al.*, 1990 ; Zuber *et al.*, 1989a), and we have shown that transfection of GAP-43 in AtT-20 cells caused the

cells to flatten and extend processes (Gamby *et al.*, 1996, manuscript#1), we investigated if transfection of [S41D]GAP-43 or [C3,4G]GAP-43 would also cause morphological changes in AtT-20 cells (figure 10). Similar to the original AtT-20 cells, the transfected cell lines grow in suspension under routine culture conditions. However, when seeded on laminin-coated plates, about 50 % of the cells transfected with [S41D]GAP-43 flattened and extended processes (figure 10d), similar to the AtT-20 cells expressing wild type GAP-43 (figure 10b). These morphological changes were not observed for the AtT-20:[C3,4G]ratGAP-43 (figure 10c) or control AtT-20:pRc/RSV CC1 (figure 10a) cell lines cultured in the same conditions.

DISCUSSION

We have shown previously that forced expression of the neuronal phosphoprotein GAP-43 in mouse anterior pituitary AtT-20 cells enhances K^+-evoked β-endorphin secretion and causes morphological changes in these cells (Gamby *et al.*, 1996, manuscript#1). Here we have used the AtT-20 cells to investigate the molecular mechanism of action of GAP-43 in these responses. We show that in the intact cell, GAP-43 is associated with CaM and that this interaction is regulated by intracellular Ca^{2+} concentration and phosphorylation. Mutant forms of GAP-43 which cannot bind CaM or associate with the plasma membrane fail to enhance K^+-evoked secretion from transfected cells. In contrast, the morphological changes caused by GAP-43 depend only on the association of GAP-43 with the plasma membrane.

GAP-43 binding to CaM and regulation of this interaction by Ca^{2+} and phosphorylation *in vivo*. The interaction between CaM and GAP-43 has been characterized extensively via *in vitro* studies, which have demonstrated that the affinity of CaM for GAP-43 is higher in the absence of Ca^{2+} that in its presence and that association of the two proteins decreased by phosphorylation of GAP-43 by PKC on ser 41, a residue in the CaM-binding domain. These results have led to the hypothesis that GAP-43 might act to sequester CaM at the plasma membrane in the unstimulated cell (Estep *et al.*, 1990). In this scenario, upon elevation of intracellular Ca^{2+}, CaM would be released and in a position to activate rapidly membrane-associated Ca^{2+}/CaM-dependent enzymes. Despite the appeal of this hypothesis, the interaction between CaM and GAP-43 has not been demonstrated in the intact cell and the physiolgical significance of this interaction remains to be established.

The intact cell cross-linking studies described here have resulted in the identification of a 70 kDa immunoreactive band that reacts with both anti-GAP-43 and anti-CaM antibodies. As this 70 kDa complex is present only where GAP-43 is expressed, it is likely that this molecular species represents a complex formed between GAP-43 and CaM. Additional evidence for this association comes from the co-immunoprecipitation of CaM with an anti-GAP-43 antibody from extracts of cross-linked cells. Previous investigations considering the abundance of these two proteins and the relative affinity of their interaction have suggested that a majority of the CaM in the intact cell would be bound to GAP-43. Our studies have provided some support for this notion, as approximately 70 % of the membrane CaM is associated with GAP-43, and the efficacy of the cross-linking is less than complete. Our findings also complement those of Gispen and colleagues, who showed that in synaptosomal plasma membrane binding of GAP-43 to exogenous radiolabeled CaM can be demonstrated after addition of a cross-linking agent (De Graan *et al.*, 1990). Finally, our demonstration that the interaction between GAP-43 and CaM is regulated by intracellular Ca^{2+} and PKC-mediated GAP-43 phosphorylation provide additional support for the hypothesis that the function of GAP-43 is to concentrate CaM at the plasma membrane and release it upon a stimulus-evoked influx of Ca^{2+}.

Experiments with primary cultures of hippocampal neurons show that here CaM and GAP-43 also formed a 70 kDa complex similar to that observed in the AtT-20 cells. Since hippocampal neurons express higher levels of GAP-43 than the transfected AtT-20 cells, a correspondingly larger fraction of cellular CaM is bound to GAP-43. Furthermore, the association of CaM and GAP-43 is also regulated by intracellular Ca^{2+} concentration and phosphorylation in the primary cultures. GAP-43 is expressed only in discrete

regions of the adult brain (Benowitz *et al.*, 1988 ; Benowitz *et al.*, 1989 ; Gispen *et al.*, 1985 ; Masliah *et al.*, 1991 ; Neve *et al.*, 1988 ; Neve *et al.*, 1987), including the hippocampus, where it is thought to be involved in synaptic plasticity and where its phosphorylation has been correlated with long-term potentiation (Chan *et al.*, 1986 ; Gianotti *et al.*, 1992 ; Lovinger *et al.*, 1985 ; Routtenberg *et al.*, 1985). Therefore sequestration of CaM may represent an vital component of the role of GAP-43 in synaptic plasticity in the mature nervous system.

Essential role of CaM binding and association with the plasma membrane in GAP-43-mediated enhancement of hormone release. The physiological significance of the GAP-43/CaM interaction at the plasma membrane in AtT-20 cells was confirmed by showing that although forced expression of wild type GAP-43 markedly increases depolarization-mediated hormone secretion, transfection of GAP-43 mutants that cannot bind CaM (S41D) or associate with the plasma membrane (C3,4G) fails to produce a significant enhancement of secretion.

In presynaptic nerve terminals, synaptic vesicles are docked in clusters at specific sites of the plasma membrane termed active zones, the proximity of which to Ca^{2+} channels permits rapid fusion of the vesicles with the plasma membrane upon an influx of Ca^{2+} (reviewed in Burns and Augustine, 1995 ; Trimble *et al.*, 1991). Similar to this specific localization of synaptic vesicles, the association of the CaM/GAP-43 complex to the plasma membrane may also function to permit the rapid secretion of neurotransmitter in response to massive stimulation, possibly via modulation .of the fusion of synaptic vesicles with the plasma membrane.

Requirement for membrane association in GAP-43-mediated morphological changes. Expression of wild type GAP-43 in AtT-20 cells causes the cells to flatten and extend processes that are stable for several days in culture. Similarly, expression of the (S41D) mutant GAP-43 also causes these morphological changes, indicating that CaM binding is not required for this effect. In contrast, AtT-20 cells expressing the (C3,4G) mutant GAP-43 resemble control cell lines morphologically, suggesting that membrane association may be necessary for the role of GAP-43 in process extension.

GAP-43 has been shown to be localized in clusters at the plasma membrane and to interact with the membrane skeleton, a prominent component of which is filamentous actin (Allsopp and Moss, 1989 ; Meiri and Gordon-Weeks, 1990 ; Moss *et al.*, 1990). GAP-43 has been shown to bind to actin *in vitro* (Strittmatter *et al.*, 1992), possibly through a C-terminal domain homologous to a portion of neurofilament NF-L (La Bate and Skene, 1989). Furthermore, in primary sensory neurons depleted of GAP-43 by antisense GAP-43 oligonucleotides, lamellar extensions of the growth cone lacked local f-actin concentrations and showed poor adhesion (Aigner and Caroni, 1995), suggesting that interaction of GAP-43 with the actin-based membrane skeleton may be important for the function of GAP-43 in the growth cone, either in the direct regulation of process extension or in the response of the growth cone to extracellular guidance cues (Strittmatter *et al.*, 1995).

EXPERIMENTAL PROCEDURES

Plasmid construction. The cDNAs encoding ([S41D]ratGAP-43; (Chapman *et al.*, 1991)) and ([C3,4G]ratGAP-43 ; (Chapman *et al.*, 1992)) were the generous gift of Dr. Dan Storm (University of Washington, Seattle). The expression vectors for these mutant forms of rat GAP-43 were produced by ligating a 750 bp HindIII/EcoRI restriction fragment containing the mutated rat GAP-43 coding sequence into the HindIII and EcoRI sites of the pRc/RSV vector (Invitrogen) with the Rous sarcoma virus (RSV) promoter (Gorman *et al.*, 1983) driving expression of the cDNA.

Cell culture and transfections. All cell culture reagents were from Life Technologies, Inc. Monolayer cultures of D16 cells and transfected cell lines were maintained in 95 % OptiMEM-I, 5 % fetal bovine serum. The original AtT-20 cells were cultured in 85 % OptiMEM-I, 10 % equine serum, 5 % fetal bovine serum ; medium for routine culture of the transfected cells contained 200 μg/ml G418. All cell lines were incubated in humidified 95 % air, 5 % CO_2 at 37 ºC. The AtT-20/D16 cells were generously provided by Dr. Lee Limbird, Department of Pharmacology, Vanderbilt University, and the original AtT-20 cells were obtained from the American Type Culture Collection (CCL89).

The original AtT-20 cells were transfected using the LipofectAMINE reagent, according to the manufacturer's instructions. Briefly, 20 x 10^6 cells were transfected with 15 μg of the GAP-43 expression plasmid and 60 μl lipofectAMINE. After 6 h of incubation at 37 ºC, the medium was removed and replaced by normal culture medium. After 72 h cells were split into selective medium containing 400 μg/ml G418. Clones were then isolated by limiting dilution and expanded in culture.

Primary cultures of rat hippocampal neurons, generously provided by Dr. Randy Hall and Andrès Barria-Roman, were prepared from rat pups 24-48 hrs post-natal. Hippocampi were dissected into room temperature osmotically balanced saline solution ("SS" : 137 mM NaCl, 5.3 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 10 mM Hepes, 33 mM glucose, 44 mM sucrose, 0.024 g/l phenol red, pH 7.3, 325 mosm), cut into 5-6- pieces and incubated for one hour with 10 ml of a 20 units/ml papain (Worthington) solution in SS. The papain solution was removed and the hippocampal fragments washed once in MEM supplemented with 10 % heat-inactivated fetal calf serum, 1 µl/ml serum extender (Collaborative Research), 21 mM glucose, 10 µg/ml 5-fluoro-2'deoxyuridine, 25 µg/ml uridine (Sigma), (complete medium). Cells were then dissociated into fresh complete medium via 15-20 passes through a pasteur pipet and plated into sterile, poly-L-lysine-coated 35-mm culture plates at an approximate density of two hippocampi per dish. Cultures were fed on post culture day 1, 4 and 7 and used for experiments on culture day 8. Cultures prepared via this method are of extremely high density (roughly 1-2 million cells per 35 mm plate) and have a very high neuron-to-glia ratio since they are cultured in the presence of a mitotic inhibitor (5-fluoro-2' deoxyuridine) from the first day.

Intact cell chemical cross-linking experiments. Cells were washed free of medium twice with Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, 5.6 mM glucose, pH 7.4) and incubated at 20 °C for the indicated time with the thiol-cleavable homobifunctional cross-linker dithiobis(succinimidyl propionate) (DSP, Pierce) at 1 mM diluted in KRH from a 25 mM stock solution in DMSO (cross-linked cells) or solvent alone (non cross-linked cells). At the end of the

incubation period, cells were transferred on ice, washed 3 times with ice-cold KRH, scraped off the dish and cell pellets were frozen at – 70 ºC until the immunoblot analysis was performed. To study the effects of calcium on the association of GAP-43 and CaM, cells were incubated for 5 min in KRH alone or KRH containing 0.1 % dimethylsulfoxide (DMSO, vehicle for A23187) as controls, or KRH containing either 10 μM calcium ionophore A23187 (Sigma), or 56 mM KCl (in which the NaCl concentration was decreased to maintain iso-osmolarity) prior to treating the cells with DSP. To study the effects of PKC phosphorylation on the association of GAP-43 and CaM, cells were incubated for 5 min in KRH containing either 1 μM tetradecanoyl phorbol acetate (TPA, Sigma) or 0.1 % DMSO (vehicle for TPA) as a control, prior to treating the cells with DSP.

Immunoprecipitation and immunoblot analysis. Subcellular fractionation was performed by lysing cells in 20 mM Tris pH 7.4, 2 mM EDTA, 1 mM EGTA and separating the particulate and soluble fractions by centrifugation at 100 000 x *g*. Proteins from the particulate fraction were solubilized with RIPA buffer (10 mM TRIS, pH 7.2, 150 mM NaCl, 1 % deoxycholate, 1 % Triton X-100, 0.1 % SDS) containing 2 μg/ml aprotinin and quantified by the method of Bradford (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

Five hundred μg of protein were immunoprecipitated with an anti-rat GAP-43 polyclonal antibody (de La Monte *et al.*, 1989) one hour on ice. Immune complexes were collected on protein-A-sepharose CL4B beads (Sigma), washed once with 1 ml of 1 M NaCl, 1 % Triton X-100, twice with 1 ml RIPA, 1 M urea and once with 1 ml 10 mM Tris, 1 mM EDTA, pH 7.5. The material bound via the cross-linking agent to the GAP-43 immune complex was released by incubating the washed beads for 30 min at 37 ºC with elution

buffer (10 mM Tris pH 7.5, 5 mM EDTA, 0.2 M dithiotreitol), followed by a brief centrifugation. The eluate containing the material released from the immune complex by the reducing agent was analyzed by immunoblot analysis with an anti-calmodulin antibody.

Proteins were resolved by SDS-polyacrylamide gel electrophoresis on SDS-12% polyacrylamide minigels (Hoefer Scientific Instruments) and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). Protein blots were blocked with 5 % non-fat milk in phosphate-buffered saline (PBS) and incubated with either an anti-rat GAP-43 polyclonal antibody (de La Monte *et al.*, 1989), an anti-GAP-43 monoclonal antibody (clone GAP-7B10, Sigma,), or an anti-calmodulin monoclonal antibody (UBI), followed by a peroxidase-conjugated secondary antibody (Sigma,). Bound antibodies were detected by enhanced chemiluminescence (Du Pont NEN) and exposure to X-ray film (X-OMAT AR, Eastman Kodak Co.). Quantitation was done by scanning exposed films with the SigmaGel analysis software (Jandel Scientific Software).

Secretion studies. Cells were plated in 6-wells cluster dishes at an initial density of 2.5×10^5 cells/well and were used for experiments 6 days later. For incubations with CRF, the medium was replaced by pre-warmed OptiMEM-I containing BSA (2.5 mg/ml) and protease inhibitors (0.1 mg/ml trypsin inhibitor, 2 μg/ml aprotinin). After 30 min the medium was removed and replaced by fresh medium without (basal) or with 100 nM CRF (Sigma) and the cells were incubated an additional 30 min at 37 °C. For K^+ stimulation, the cells were equilibrated for 15 min in KRH. The medium was then removed and the cells were incubated for 5 min either in KRH buffer (basal), or in KRH buffer containing 56 mM KCl (in which the NaCl concentration was decreased

to maintain iso-osmolarity). After the incubation with either CRF or elevated potassium the medium was collected, centrifuged 3 min at 1 700 x *g* to remove dislodged cells and debris and phenylmethylsulfonylfluoride added to a final concentration of 2 mM. The cells were collected in PBS, centrifuged and protein was extracted from the cell pellets with RIPA buffer containing protease inhibitors. Secreted and cellular hormones were measured by radioimmunoassay (RIA). Net secretion (CRF- or K^+-stimulated minus basal) was expressed as the percent of total cellular stores of ß-endorphin released during the incubation period.

Radioimmunoassay. β-endorphin immunoassays were performed as described previously (Hatfield *et al.*, 1988), using an antiserum which is specific for ß-endorphin residues 15-26. Synthetic acetyl-β-endorphin 1-27 was used as tracer and standard, and a 12-point standard curve was assayed with each group of samples. The unpaired 't' test was used to determine the statistical significance of the results.

Intracellular Ca^{2+} measurements. Cells were collected by centrifugation and re-suspended at 10^7 cell/ml in KRH buffer. Fura-2/acetoxy-methyl ester (fura2/AM, Molecular Probes) was added at a final concentration of 1 μM and loading was done for 30 min in the dark at room temperature. Unincorporated dye was removed by washing the cells once with KRH. Cells were re-suspended at 0.5 x 10^6 cells/ml in KRH pre-warmed at 37 ºC and transferred in fluorometer cuvette and incubated at 37 ºC for 30 min, to allow complete de-esterification. Fura-2 fluorescence was measured using a LS50 luminescence spectrometer (Perkin-Elmer) and Ca^{2+} concentrations were calculated as described by Grynkiewicz *et al.* (Grynkiewicz *et al.*, 1985), using the Intracellular Biochemistry software package (Perkin-Elmer).

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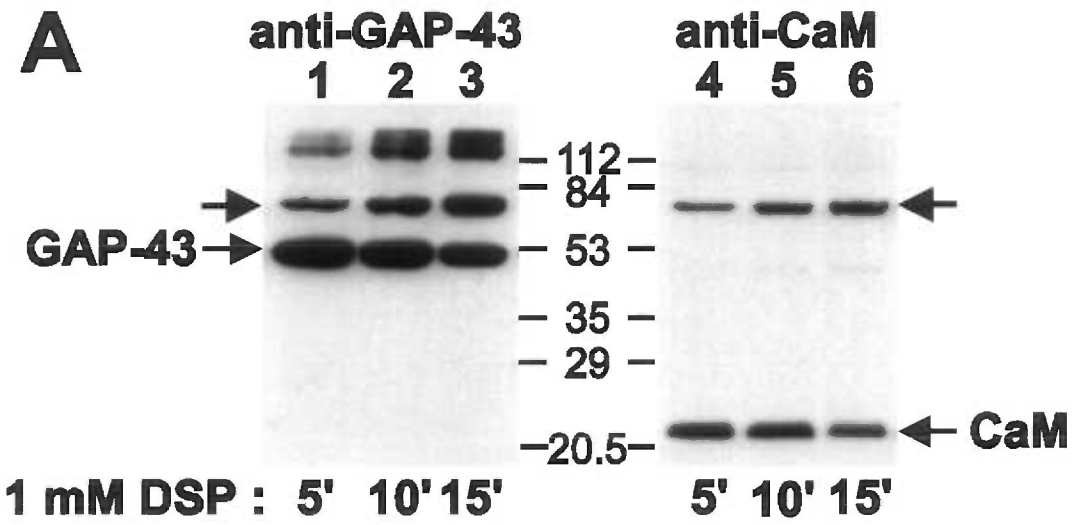
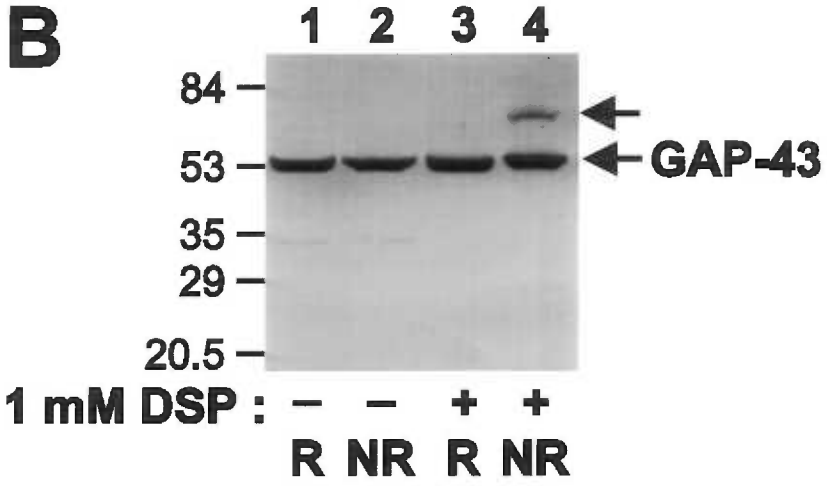
Figure 1

Immunoblot analysis of crosslinked cells extracts. AtT-20/D16 cells were treated with 1 mM DSP (crosslinked) or DMSO (control) in KRH. Subcellular fractionation was performed as described under "Experimental Procedures" and 20 μg of membrane proteins were analyzed on a 12 % acrylamide-SDS gel, followed by immunoblot analysis. Panel A, immunoblot with an anti-GAP43 polyclonal antibody. Control cells : reducing conditions (lane 1, R), non-reducing conditions (lane 2, NR) ; Crosslinked cells : reducing conditions (lane 3), non-reducing conditions (lane 4). Panel B, immunoblot with an anti-CaM monoclonal antibody. Control cells : non-reducing conditions (lane 1), reducing conditions (lane 2) ; Crosslinked cells : non-reducing conditions (lane 3), reducing conditions (lane 4).


A
anti-GAP-43
anti-CaM
1 2 3
4 5 6
112
84
53
35
29
20.5
GAP-43
CaM
1 mM DSP : 5' 10' 15'
5' 10' 15'


B
1 2 3 4
84
53
35
29
20.5
GAP-43
1 mM DSP : – – + +
R NR R NR

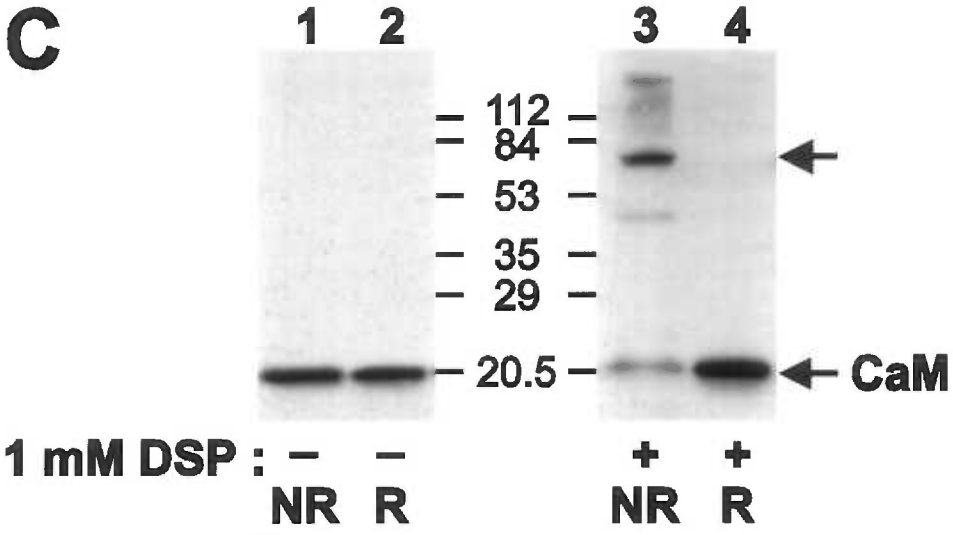

C
1 2
3 4
112
84
53
35
29
20.5
CaM
1 mM DSP : – – + +
NR R NR R

Figure 2

The 70 kDa complex is absent from cells lacking GAP-43. D16 cells, expressing GAP-43 or AtT-20 cells, lacking GAP-43 were treated with 1 mM DSP in KRH, followed by subcellular fractionation as described under "Experimental Procedures". Twenty μg of membrane (m) or soluble (s) proteins were analyzed on a 12 % acrylamide-SDS gel in non-reducing conditions, followed by immunoblot analysis with an anti-GAP-43 monoclonal antibody (lanes 1-4) or with an anti-CaM monoclonal antibody (lanes 5-8). Membrane fraction from D16 cells (lanes 1 & 5) ; Soluble fraction from D16 cells (lane 2 & 6) ; membrane fraction from AtT-20 cells (lane 3 & 7) ; soluble fraction from AtT-20 cells (lanes 4 & 8).

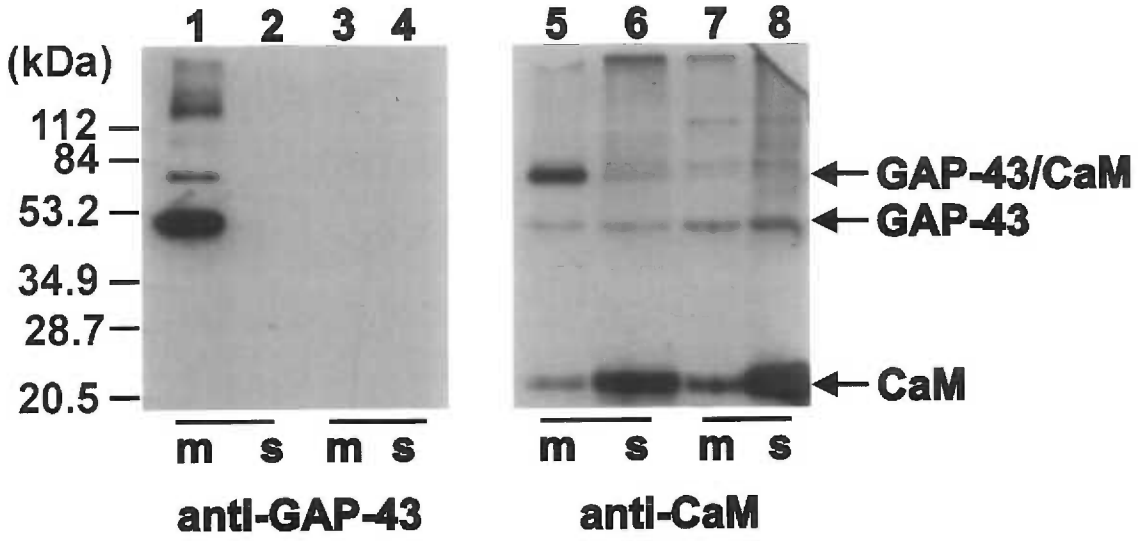

1 2 3 4
5 6 7 8
(kDa)
112
84
53.2
34.9
28.7
20.5
GAP-43/CaM
GAP-43
CaM
m s m s
m s m s
anti-GAP-43
anti-CaM

Figure 3

CaM coimmunoprecipitates with GAP-43. D16 cells, expressing GAP-43 were treated with 1 mM DSP (crosslinked) or DMSO (control) in KRH, followed by subcellular fractionation as described under "Experimental Procedures". Five hundred μg or membrane (m) or soluble (s) proteins were immunoprecipitated with an anti-GAP-43 polyclonal antibody. Eluates from the immune complex were analyzed on a 15 % acrylamide-SDS gel, followed by immunoblot analysis with an anti-CaM antibody. Crosslinked cells : lane 1, membrane fraction ; lane 2, soluble fraction. Control cells : lane 3, membrane fraction ; lane 4, soluble fraction.

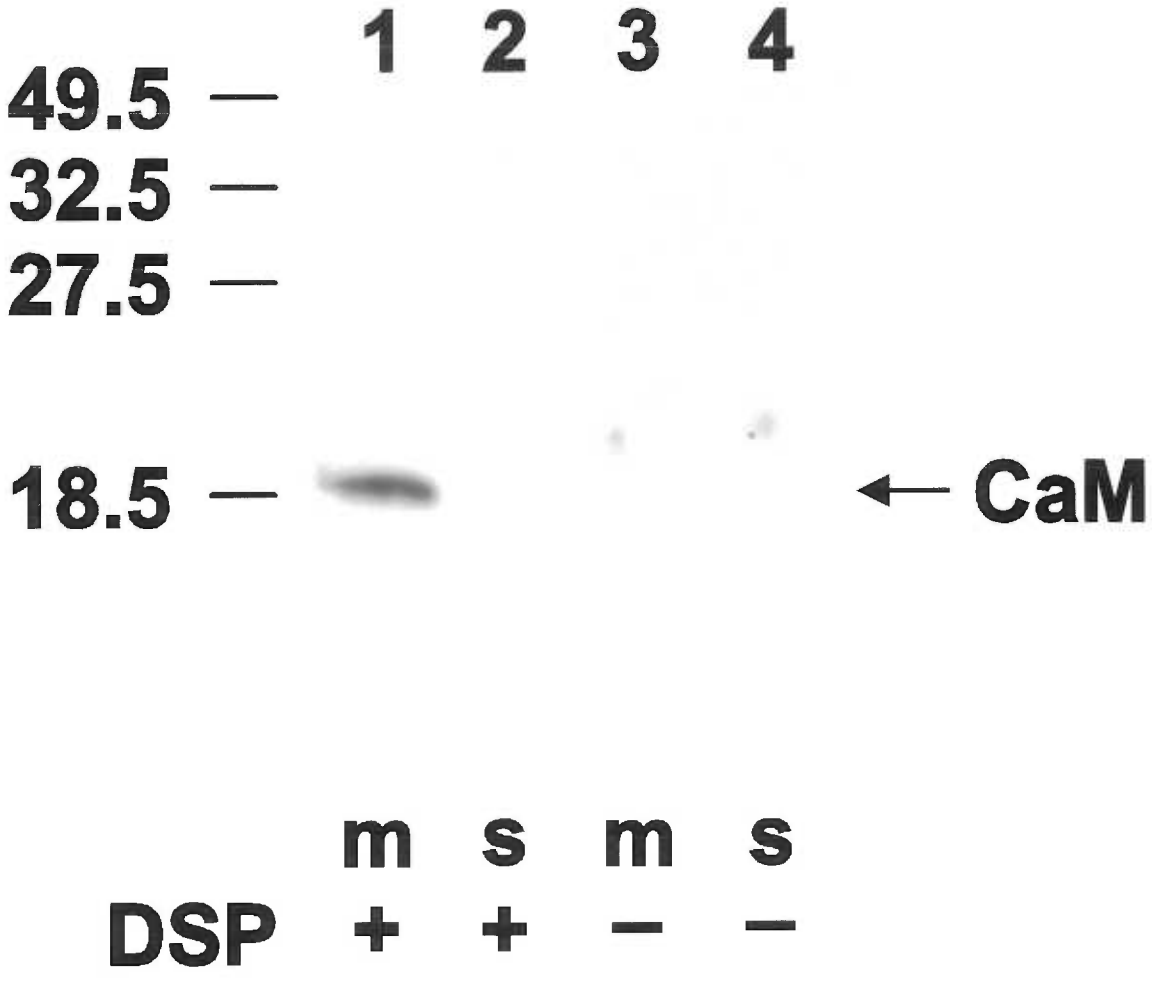

1 2 3 4
49.5
32.5
27.5
18.5
CaM
m s m s
DSP + + − −

Figure 4

Intracellular calcium elevation or PKC phosphorylation of GAP-43 disrupt the association between GAP-43 and CaM. D16 cells were treated with the indicated agonist for 5 min, followed by crosslinking with 1 mM DSP for 5 min. Subcellular fractionation was performed as described under "Experimental Procedures" and 20 μg of membrane proteins were analyzed on a 12 % acrylamide-SDS gel in non-reducing conditions, followed by immunoblot analysis with an anti-GAP-43 monoclonal antibody (lanes 1 & 2) or an anti-CaM monoclonal antibody (lanes 3 & 4). Panel A, treatment with 10 μM Ca^{2+} ionophore A23187 (lanes 1 & 3), or DMSO (vehicle for A23187 ; lane 2 & 4). Panel B, treatment with KRH (control, lanes 1 & 3) or 56 mM KCl (lane 2 & 4). Panel C, treatment with DMSO (vehicle for TPA ; lane 1 & 3) or 1 μM TPA (lanes 2 & 4).

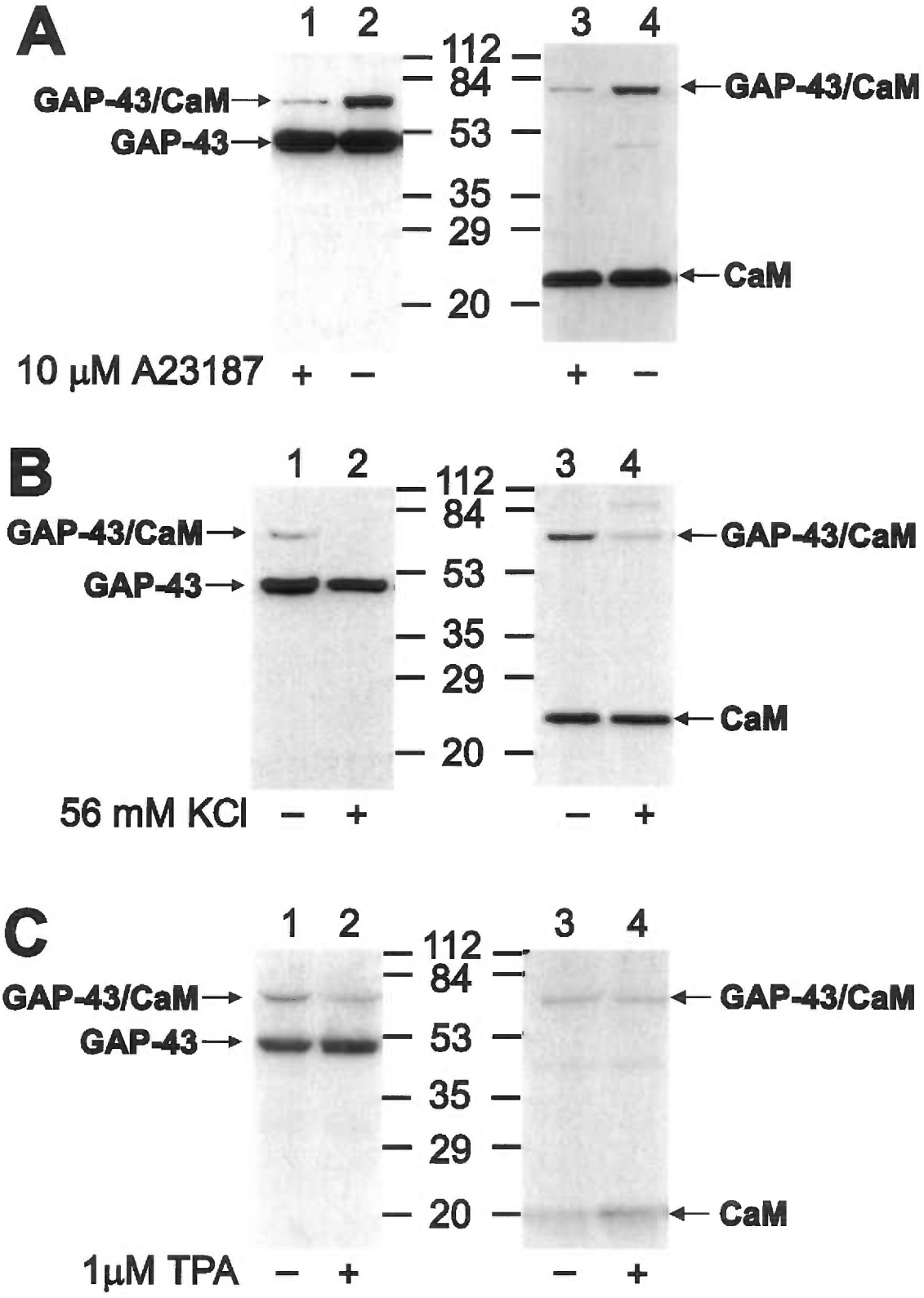

A
1 2 3 4
GAP-43/CaM
GAP-43
112
84
53
35
29
20
GAP-43/CaM
CaM
10 μM A23187 + − + −
B
1 2 3 4
GAP-43/CaM
GAP-43
112
84
53
35
29
20
GAP-43/CaM
CaM
56 mM KCl − + − +
C
1 2 3 4
GAP-43/CaM
GAP-43
112
84
53
35
29
20
GAP-43/CaM
CaM
1μM TPA − + − +

Figure 5

In primary cultures of hippocampal neurons the association of GAP-43 and CaM is also regulated by calcium and phosphorylation. Primary cultures of hippocampal neurons (8 days post-culture) were treated in the same conditions as for figure 5. Twenty μg of total cell extracts were analyzed on a 12 % acrylamide-SDS gel in non-reducing conditions, followed by immunoblot analysis with an anti-GAP-43 monoclonal antibody (panel A) or an anti-CaM monoclonal antibody (panel B). Lanes 1, treatment with DMSO (vehicle for A23187 and TPA) ; lanes 2, treatment with 1 μM TPA, lanes 3, treatment with 10 μM A23187 ; lanes 4, treatment with 56 mM KCl for 5 min ; lanes 5, treatment with 56 mM KCl for 1 min ; lanes 6, control KRH.

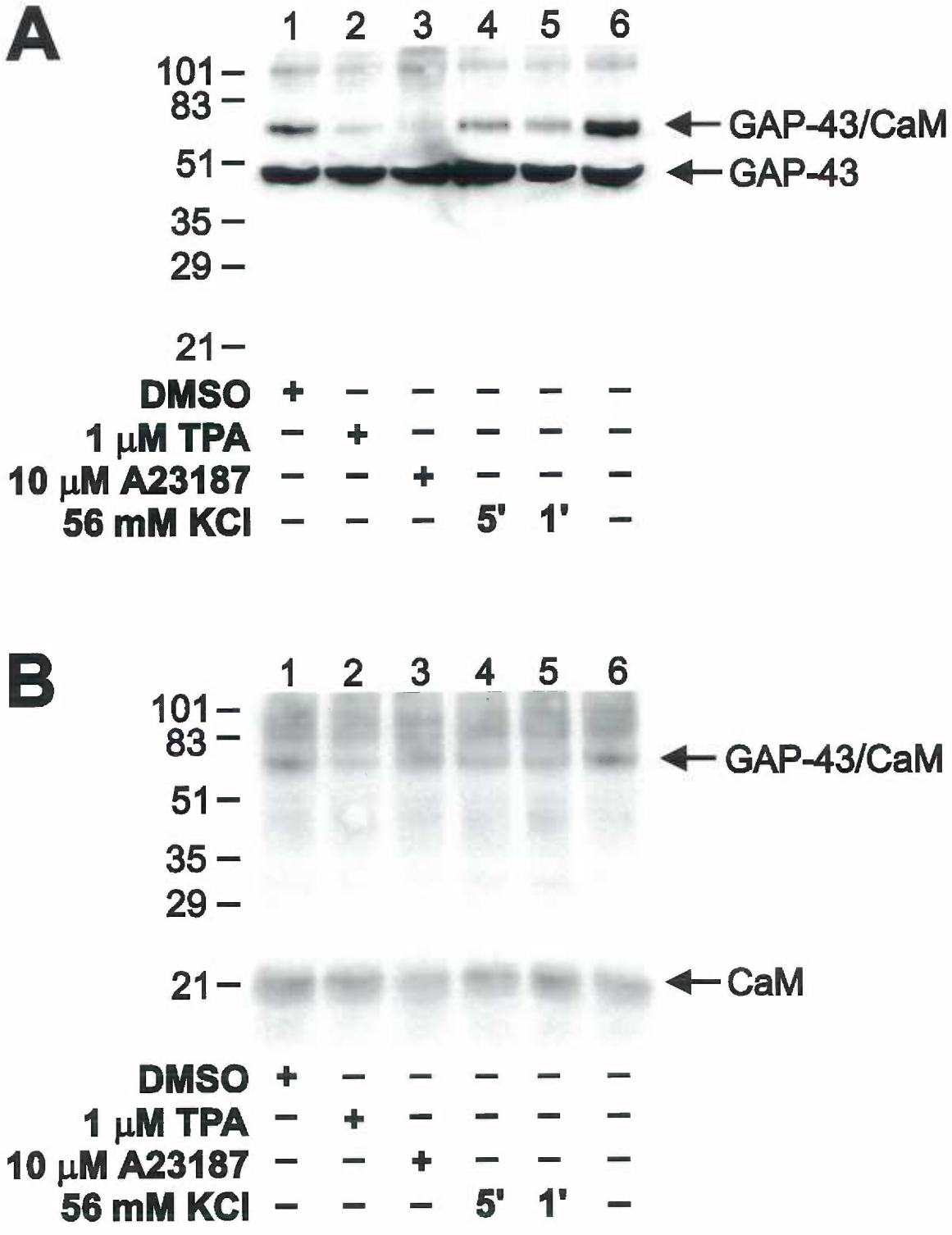

A
1 2 3 4 5 6
101
83
51
35
29
21
GAP-43/CaM
GAP-43
DMSO + – – – – –
1 μM TPA – + – – – –
10 μM A23187 – – + – – –
56 mM KCl – – – 5' 1' –
B
1 2 3 4 5 6
101
83
51
35
29
21
GAP-43/CaM
CaM
DMSO + – – – – –
1 μM TPA – + – – – –
10 μM A23187 – – + – – –
56 mM KCl – – – 5' 1' –

Figure 6

CaM is required for K^+-evoked secretion. K^+-evoked secretion was measured as described under "Experimental Procedures". Prior to treatment, D16 cells were incubated for 30 min KRH solution containing the indicated dose of CaM antagonist. Cells were then depolarized for 5 min in the continued presence of the CaM antagonist. β-endorphin release was measured by radioimmunoassay as described under "Experimental Procedures". Panel A, dose-response of calmidazolium chloride ; Panel B, dose-response of trifluoperazine dimaleate ; Panel C, dose-response of W7. Experiments were done in triplicate.

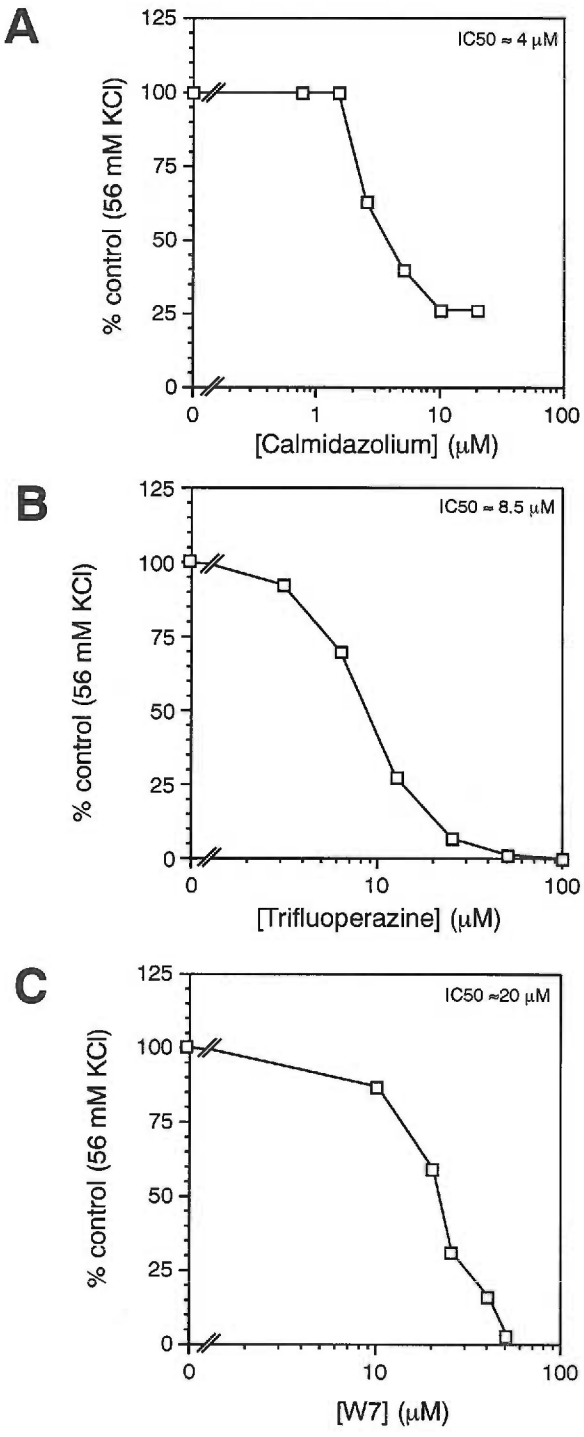

A
IC50 ≈ 4 μM
% control (56 mM KCl)
125
100
75
50
25
0
0
1
10
100
[Calmidazolium] (μM)
B
IC50 ≈ 8.5 μM
% control (56 mM KCl)
125
100
75
50
25
0
0
10
100
[Trifluoperazine] (μM)
C
IC50 ≈20 μM
% control (56 mM KCl)
125
100
75
50
25
0
0
10
100
[W7] (μM)

Figure 7

Expression of mutant GAP-43 in the transfected AtT-20 cells. Original AtT-20 were transfected with the expression plasmid for either [S41D]ratGAP-43 or [C3,4G]ratGAP-43 and stably transformed cell lines were selected as described under "Experimental Procedures". Twenty μg of membrane (m) or soluble (s) proteins were resolved on a 12 % SDS/polyacrylamide gel and immunoblot analysis was performed as described previously with a monoclonal anti-GAP-43 antibody. Panel A, lanes 1, AtT-20:[S41D]ratGAP-43 R4B ; lanes 2, AtT-20:[S41D]ratGAP-43 R5D ; lanes 3, AtT-20:[S41D]ratGAP-43 T3G ; lanes 4, AtT-20:[S41D]ratGAP-43 U2E ; lanes 5, AtT-20:[S41D]ratGAP-43 U4C. Panel B, lanes 1, AtT-20:[C3,4G]ratGAP-43 H2 ; lanes 2, AtT-20:[C3,4G]ratGAP-43 I1 ; lanes 3, AtT-20:[C3,4G]ratGAP-43 J2 ; lanes 4, AtT-20:[C3,4G]ratGAP-43 L1 ; lanes 5, AtT-20:[C3,4G]ratGAP-43 M5G.

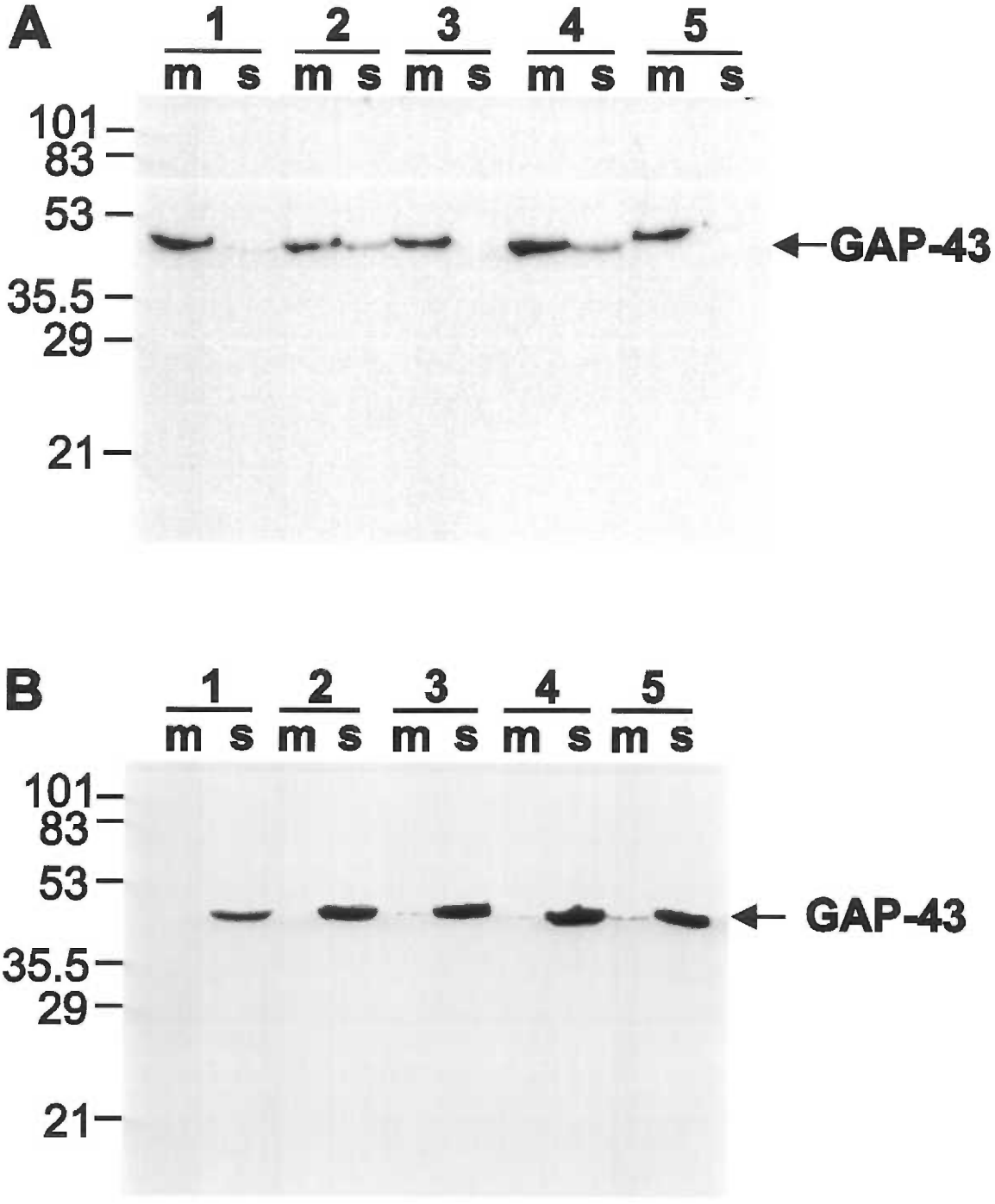

A
1 2 3 4 5
m s m s m s m s m s
101
83
53
35.5
29
21
GAP-43
B
1 2 3 4 5
m s m s m s m s m s
101
83
53
35.5
29
21
GAP-43

Figure 8

Association of CaM with GAP-43 in mutants. AtT-20:[S41D]ratGAP-43 or AtT-20:[C3,4G]ratGAP-43 cells were crosslinked for 30 min with 1 mM DSP. Subcellular fractionation was performed as described previously, 20 μg proteins were resolved on a 12 % acrylamide-SDS gel in non-reducing conditions, followed by immunoblot analysis with anti-GAP-43 (lanes 1) or anti-CaM (lanes 2) monoclonal antibodies. Panel A, membrane proteins from crosslinked AtT-20:[S41D]ratGAP-43 cells. Panel B, soluble proteins from crosslinked AtT-20:[C3,4G]ratGAP-43.

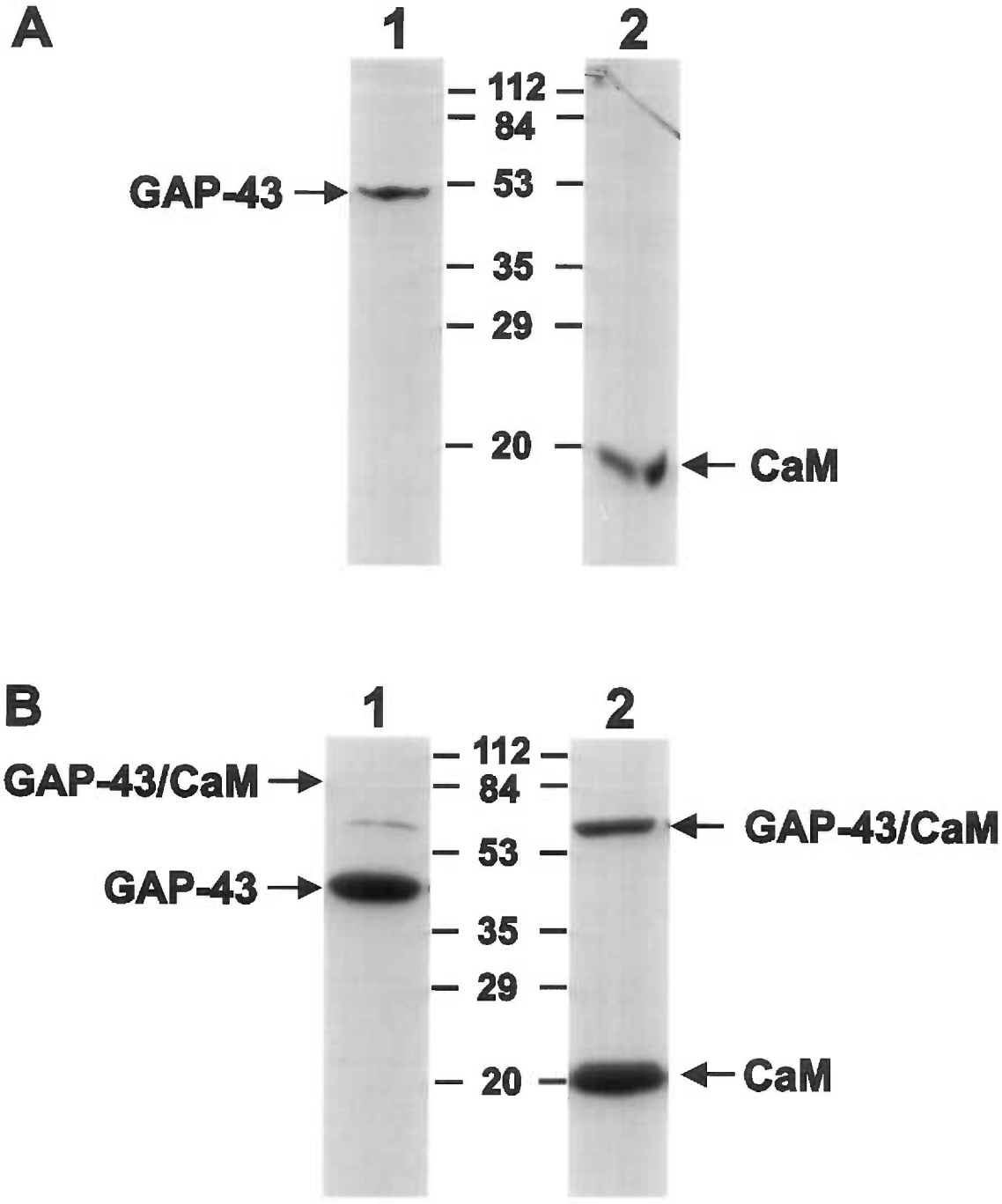

A
1
2
112
84
GAP-43
53
35
29
20
CaM
B
1
2
GAP-43/CaM
112
84
GAP-43/CaM
53
GAP-43
35
29
20
CaM

TABLE I

Net potassium- or CRF-stimulated secretion of β-endorphin from transfected AtT-20 clones

Stimulation of secretion was determined by measuring basal and CRF- or K^+-evoked release of β-endorphin as described under "Experimental Procedures". Basal release was on the average 8.5 ± 1.0 % in 30 min or 1.8 ± 0.4 % in 5 min for AtT-20 : [C3,4G]ratGAP-43 clones and 6.6 ± 1.3 % in 30 min or 2.6 ± 0.3 % in 5 min for AtT-20 : [S41D]ratGAP-43 clones.

Mutant GAP-43	Clone number	net K^+-evoked secretion	net CRF-evoked secretion
[C3,4G]rat GAP-43	H2	1.9 ± 0.5	10.9 ± 6.3
	I1	2.8 ± 1.4	8.3 ± 3.1
	J2	2.9 ± 0.9	8.6 ± 1.8
	M5G	2.6 ± 0.9	7.8 ± 1.6
	L1	3.0 ± 2.1	7.4 ± 2.7
	average	2.6 ± 0.4	8.6 ± 1.4
[S41D]rat GAP-43	R4B	2.1 ± 1.1	7.1 ± 0.9
	R5D	3.0 ± 1.5	12.3 ± 4.0
	T3G	2.4 ± 0.7	7.2 ± 2.3
	U2E	2.8 ± 1.9	9.9 ± 2.9
	U4C	3.6 ± 1.2	6.7 ± 1.4
	average	2.8 ± 0.6	8.6 ± 2.4

Net secretion (CRF- or K^+- minus basal) is expressed as the percent of total cellular stores of β-endorphin ; values are mean ± S. D. of at least 3 determinations done in triplicate.

Figure 9

K^+-evoked influx of calcium inAtT-20 cells transfected with mutant GAP-43. Cells were loaded with the fluorescent dye fura2/AM. At the time indicated by the arrow, KCl was added to the cells to a final concentration of 56 mM and intracellular calcium was measured as described under Experimental Procedures. Panel A, intracellular calcium concentration in AtT-20:[S41D]ratGAP-43 T3G cells. Panel B, intracellular calcium concentration in AtT-20:[C3,4G]ratGAP-43 H2 cells. The graphs shown are representative of K^+-evoked Ca^{2+} influx in AtT-20:[S41D]ratGAP-43 and AtT-20:[C3,4G]ratGAP-43 cell lines. At least 3 independent determinations were performed for each cell line.

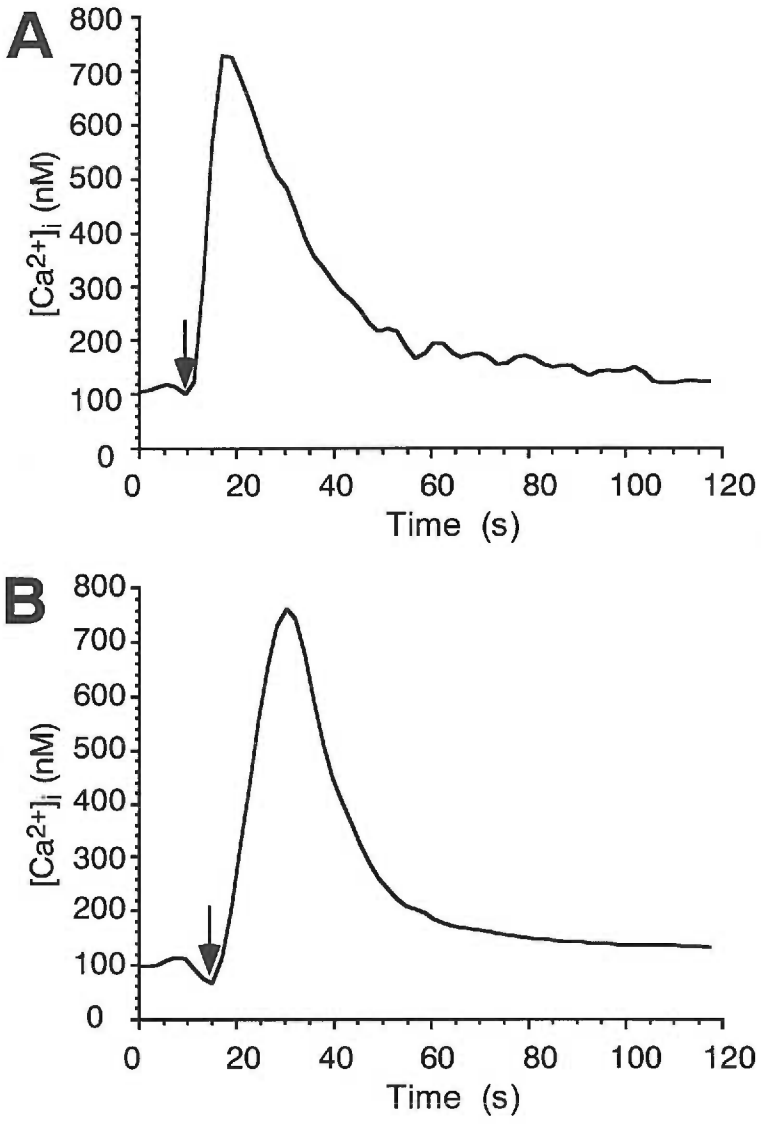
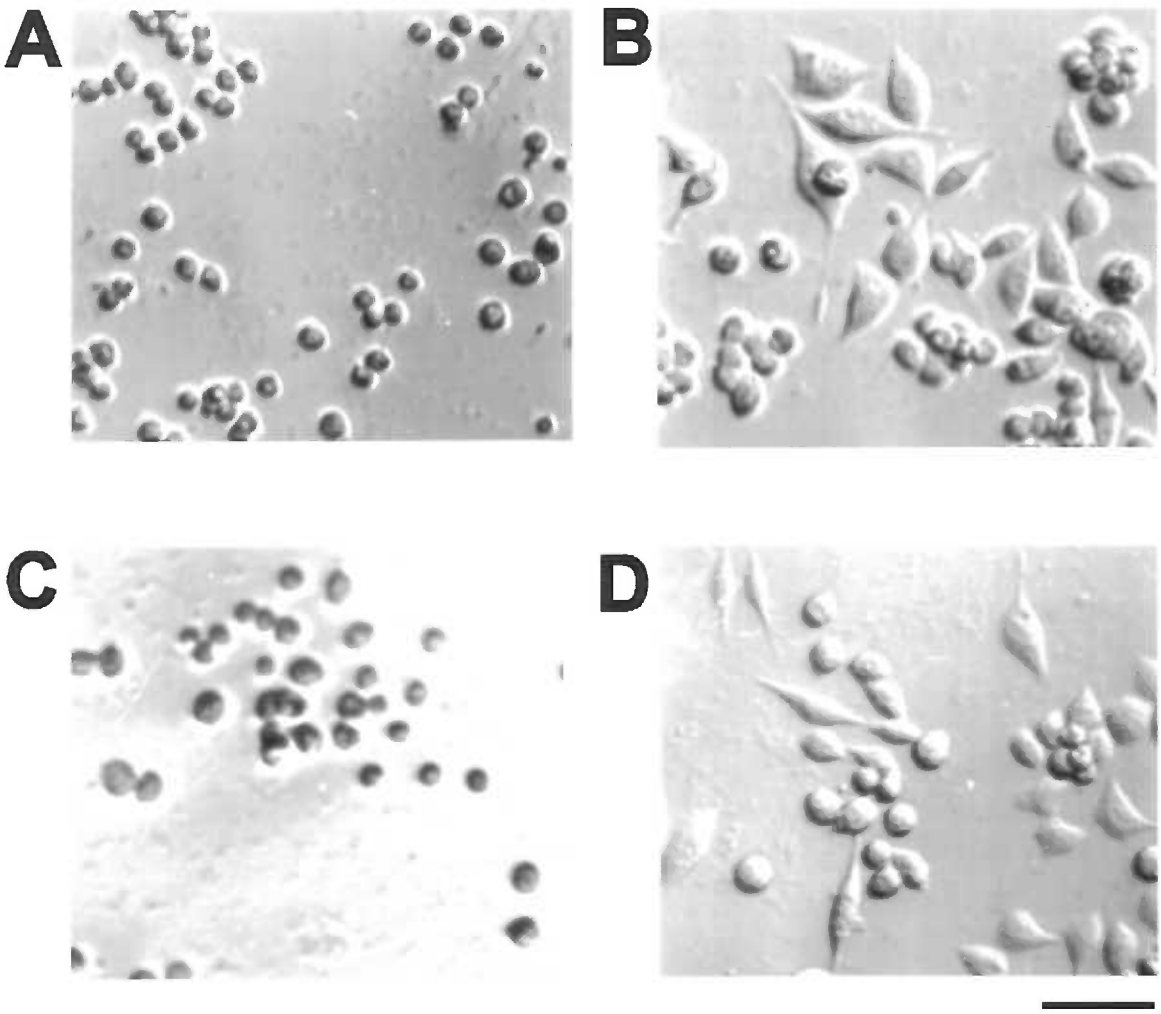

A
800
700
600
500
400
300
200
100
0
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (s)
B
800
700
600
500
400
300
200
100
0
[Ca2+]i (nM)
0
20
40
60
80
100
120
Time (s)

Figure 10

GAP-43 induced morphological changes require association of GAP-43 with the plasma membrane but binding of CaM. AtT-20:pRc/RSV CC1 control cells (panel A), AtT-20:ratGAP-43 G8D (wild type GAP-43, panel B), AtT-20:[C3,4G]ratGAP-43 M5G (panel C) or AtT-20:[S41D]ratGAP-43 R4B (panel D) were plated at an initial density of 10^5 cells/plate on 35-mm plates coated with laminin (10 μg/plate) and cultured for 3 days before fixation. Scale bar, 50 μm.


A
B
C
D

DISCUSSION

GAP-43 has been shown to be involved in physiological functions of the growth cone an nerve terminal, such as axon elongation, synaptic plasticity and modulation of second messenger pathways. Despite numerous studies, the precise role of GAP-43 in these processes remained unclear. The objective of this thesis was to analyze the role of GAP-43 in exocytosis and determine domains of the protein important for its function, thus providing informations on the mechanism of GAP-43 action.

We have established a cell culture model system, based on the mouse anterior pituitary tumor AtT-20 cell line. These cells secrete peptide hormones derived from the proopiomelanocortin precursor in equimolar amounts. AtT-20 cells have been extensively used to study hormone secretion and the various pathways controlling secretion have been well characterized. For example, it has been shown that corticotrophin-releasing factor, the physiological agonist for corticotropes and membrane-depolarization stimulate secretion from AtT-20 cells. As in other cell culture model systems a correlation between the level of GAP-43 expression and neurotransmitter release had been demonstrated, our initial observation that the original AtT-20 cells did not express GAP-43, whereas it was expressed at high levels in a subclone designated as D16 prompted us to compare hormone secretion from the two cell lines. Membrane-depolarization evoked little hormone secretion from original AtT-20 cells but produced a marked stimulation of secretion from the D16 cells. Thus, the level of GAP-43 expression is correlated with hormone secretion in the AtT-20 cells. The difference in the response to membrane-depolarization could not be explained by a lack of calcium influx or a defect in the secretory machinery in the original AtT-20 cells, since

membrane depolarization of the AtT-20 cells elicited a calcium influx similar to that in the D16 cells and original AtT-20 cells secreted normal amounts of β–endorphin in response to CRF. Furthermore, expression of wild type rat GAP-43 in the original AtT-20 enhanced K^+-evoked β–endorphin secretion. Our results are in agreement with those of others who have demonstrated a role of GAP-43 in hormone or neurotransmitter release by blocking GAP-43 with antibodies or inhibiting GAP-43 expression with antisense RNA and showing that either treatment caused a reduction of neurotransmitter or hormone release (Dekker *et al.*, 1989 ; Dekker *et al.*, 1991 ; Dekker *et al.*, 1991 ; Neve *et al.*, 1992 ; Hens *et al.*, 1993a ; Ivins *et al.*, 1993 ; Hens *et al.*, 1995). However, the forced expression of GAP-43 in AtT-20 cells causing enhancement of K^+-evoked secretion was the first demonstration of a positive effect of GAP-43 on exocytosis, thus providing a valuable model system to analyze the mechanism of GAP-43 action.

It has been demonstrated that the affinity of calmodulin for GAP-43 is higher in the absence of Ca^{2+} than in its presence and decreased by phosphorylation of GAP-43 by PKC on ser^{41}, a residue in the calmodulin-binding domain (Andreasen *et al.*, 1981 ; Andreasen *et al.*, 1983 ; Cimler *et al.*, 1985 ; Alexander *et al.*, 1987 ; Chapman *et al.*, 1991). However, the interaction between calmodulin and GAP-43 had not been demonstrated *in vivo*. Intact cell crosslinking experiments with the cell-permeable crosslinker DSP in AtT-20 cells expressing wild type GAP-43 and co-immunoprecipitation of calmodulin with GAP-43 demonstrated that calmodulin is associated with GAP-43 *in vivo*. At least to 70 % of the membrane calmodulin is associated with GAP-43, thus GAP-43 appears to be the major calmodulin binding protein at the plasma membrane. These results are in agreement with the

conclusions of Storm and colleagues who showed that the affinity of calmodulin for GAP-43 and the abundance of GAP-43 in the brain are such that GAP-43 could be complexed to most or all of the calmodulin in unstimulated cells (Alexander *et al.*, 1987), and to the results of Gispen and colleagues, who showed that in synaptosomal plasma membrane GAP-43 binds to exogenously added calmodulin in the absence of Ca^{2+} to form a 70 kDa complex upon addition of a crosslinking agent (De Graan *et al.*, 1990). Moreover, our experiments provide the first demonstration of the binding of calmodulin to GAP-43 *in vivo*. We also showed that the interaction between GAP-43 and calmodulin is regulated by an influx of Ca^{2+} caused by the action of a Ca^{2+} ionophore or by membrane depolarization.

Further studies in the synaptosomes blocking GAP-43 with antibodies targeted to the N-terminus of the protein have suggested that the calmodulin-binding domain might be important for GAP-43 function (Hens *et al.*, 1995). The physiological relevance of the association between calmodulin and GAP-43 for GAP-43 function was demonstrated by the fact that the expression in AtT-20 cells of a mutant GAP-43 which cannot associate with calmodulin (S41D) failed to enhance K^+-evoked hormone secretion. Expression in AtT-20 of a mutant GAP-43 which binds to calmodulin but does not associate with the plasma membrane also gave the same result. Expression of wild type or mutant GAP-43 in AtT-20 cells however had no effect on CRF-evoked hormone release. This may be due to the activation of different pathways by CRF or membrane depolarization which have been extensively characterized in the AtT-20 cells. CRF, binds to cell surface receptor coupled to GTP-binding proteins (Gs) and causes an elevation of cAMP and activation of the cAMP-dependent protein kinase. Activation of

voltage-gated calcium channels by phosphorylation results in the elevation of intracellular calcium within minutes after the addition of CRF. In contrast, K^+ depolarization produces a more rapid and larger influx of calcium through voltage-gated calcium channels without an increase in intracellular cAMP. Thus, GAP-43 by sequestering calmodulin at the inner face of the plasma membrane and releasing calmodulin upon an influx of Ca^{2+} may be critical for facilitating the activation of calcium-dependent processes following the transient elevation of intracellular calcium. The most attractive candidate for mediating calcium-dependent processes enhancing neurotransmitter release is the calcium/calmodulin-dependent protein kinase II (CaM-kinase II). CaM-kinase II is present in presynaptic terminals. Injection of CaM-kinase II into the giant squid axon has been shown to increase depolarization-evoked neurotransmitter release (Llinás *et al.*, 1985). In isolated synaptosomes, K^+ depolarization causes the activation of CaM-kinase II, phosphorylation of synapsin I and increased neurotransmitter release (Gorelick *et al.*, 1988). Synapsin I is a vesicle-associated protein anchoring synaptic vesicles to the actin filament. The phosphorylation by CaM-kinase II of synapsin I has been shown to reduce the affinity of synapsin I for synaptic vesicles and the bundling of f-actin by synapsin I (reviewed in Trimble *et al.*, 1991). Thus, Ca^{2+}-dependent phosphorylation of synapsin I may regulate the availability of vesicle by releasing vesicles from the actin network and also to facilitate access to these vesicle to the site of exocytosis by promoting the disassembly of the actin network.

The role of GAP-43 in exocytosis may be relevant to its function in the adult brain. GAP-43 expression dramatically decreases as the nervous system matures but remains elevated in regions of the brain associated with learning

and memory (Gispen *et al.*, 1985 ; Neve *et al.*, 1987 ; Benowitz *et al.*, 1988 ; Neve *et al.*, 1988 ; Benowitz *et al.*, 1989 ; Masliah *et al.*, 1991). GAP-43 expression remains high in nerve terminals of the hippocampus, where it is thought to be involved in synaptic plasticity and where its phosphorylation has been correlated with long-term potentiation (Lovinger *et al.*, 1985 ; Routtenberg *et al.*, 1985 ; Chan *et al.*, 1986 ; Gianotti *et al.*, 1992). Long-term potentiation involves an increase in synaptic transmission efficiency, in part due to the increased release of neurotransmitter from the presynaptic terminus (Bekkers and Stevens, 1990 ; Malinow and Tsien, 1990) We have demonstrated that in hippocampal neurons, K^+-depolarization or the stimulation of PKC results in the release of calmodulin from GAP-43. Thus, in the presynaptic terminal, GAP-43 may contribute to the induction of LTP by modulating Ca^{2+}/calmodulin-dependent processes and enhancing neurotransmitter release. In addition to influencing the availability and/or access of vesicles to the plasma membrane through the modulation of Ca^{2+}/calmodulin-dependent processes, GAP-43 may have a more direct effect on controlling the access of vesicle, through interaction with the actin filament. GAP-43 has been shown to be localized in clusters at the plasma membrane and to interact with the membrane skeleton, where filamentous actin is a prominent component (Allsopp and Moss, 1989 ; Meiri and Gordon-Weeks, 1990 ; Moss *et al.*, 1990). GAP-43 has been shown to interact with filamentous actin (Strittmatter *et al.*, 1992), although this interaction is not well characterized. Thus, by interacting with the actin filament, which forms a barrier that holds the docked vesicles a few nm away from the plasma membrane, GAP-43 may facilitate the access of vesicles to the site of fusion. Alternatively, the interaction of GAP-43 with actin may only be necessary for specific localization of the protein in close proximity of targets for the delivery

of calmodulin. An extension of the present work may be to characterize the domain of GAP-43 involved in actin binding and to investigate the effect on secretion of mutant GAP-43 which cannot bind to actin.

In contrast to a number of studies, including the present work, which suggest that GAP-43 is enhancing neurotransmitter or hormone release, under different experimental conditions GAP-43 has been shown to inhibit the release of hormone from chromaffin cells. In that study however, non-palmitylated GAP-43 peptides were added to permeabilized cells, and blocked secretion by activating GTP-binding proteins (Vitale *et al.*, 1994). The 10 N-terminal amino acids of GAP-43 have been shown to stimulate GTP/GDP exchange on the α subunit of G_o or G_i (Strittmatter *et al.*, 1990 ; Strittmatter *et al.*, 1991 ; Strittmatter *et al.*, 1994a). The free sulfhydryl groups of the cysteine residues appear to be crucial for GAP-43 activation of G-proteins since replacement of cys^3 and cys^4 by threonine residues or palmitylation of these 2 cysteines abolishes this activity (Strittmatter *et al.*, 1990 ; Sudo *et al.*, 1992). Intriguingly, in D16 cells transfected with wild type GAP-43, forced overexpression of GAP-43 resulted in the partial localization of GAP-43 in the cytosol. The large amount of soluble GAP-43 in these cells was not palmitylated, possibly because of saturation of the machinery responsible for this postranslational modification. Furthermore, evoked secretion from these cells was greatly diminished, compared to that from the parent D16 cells, possibly by activation of G-proteins which has been shown to inhibit secretion from AtT-20 cells (Law *et al.*, 1991). Thus, under certain conditions that cause depalmitylation of GAP-43, the protein might impinge upon a different, G-protein-mediated pathway and exert an inhibitory effect on exocytosis. This suggest that the role of GAP-43 in the modulation of secretion is likely to be

complex. The AtT-20 cells may also provide an useful model system for the study of the interaction with G-proteins and its functional significance.

Expression of wild type GAP-43 in AtT-20 cells causes the cells to flatten and extend processes which remain present for at least several days. These results are consistent with a number of studies where expression of GAP-43 in non-neuronal cells was shown to cause neurite elongation. In some cases however, elongation of neurite did not depend on the presence of GAP-43. For example a PC12 cell line that lacked GAP-43 was still able to extend processes in response to NGF (Baetge and Hammang, 1991). In DRG neurons, injection of GAP-43 enhanced growth cone collapse and neurite retraction induced by serotonin, presumably by activating GTP-binding proteins (Igarashi *et al.*, 1995). Disruption of the GAP-43 gene in transgenic mice did not prevent axon elongation but growth cones of retinal failed to interpret axonal guidance cues from their environment (Strittmatter *et al.*, 1995). These results suggests that rather than being necessary for the structure of the growth cone, GAP-43 may act to regulate the sensitivity of growth cones to extrinsic signals. However, in AtT-20 cells, expression of a mutant GAP-43 which cannot bind to calmodulin also flatten and extended processes. In contrast, AtT-20 cells expressing a mutant GAP-43 which cannot bind to the plasma membrane resemble control AtT-20 cells transfected with the vector alone and do not elongate processes, suggesting that membrane association may be necessary for the role of GAP-43 in neurite elongation. Furthermore, in primary sensory neurons depleted of GAP-43 by antisense GAP-43 oligonucleotides, the lamellar extensions of the growth cone lacked local f-actin concentrations and showed poor adhesion (Aigner and Caroni, 1995), suggesting that interaction of GAP-43 with the actin filament may also be

important for the function of GAP-43 in the growth cone. GAP-43 association with the plasma membrane through its N-terminal domain places the C-terminal region of this extended protein in proximity of the actin membrane skeleton. Therefore the association of GAP-43 with the plasma membrane may be critical for interacting with f-actin and regulating process extension. AtT-20 cells may also be useful to analyze the effect of expression of mutant forms of GAP-43 which cannot bind actin on cell morphology.

While some issues about the precise role of GAP-43 in exocytosis and neurite elongation clearly remain to be resolved, our studies have identified domains of the protein that are critical for its function in these processes and pointed to possible pathways that GAP-43 might modulate.

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