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Evidence for a Role of Calmodulin in Calcium-Induced Noradrenaline Release from Permeated Synaptosomes: Effects of Calmodulin Antibodies and Antagonists

Jacques J. H. Hens, A. Beate Oestreicher, Marina De Wit, Arnoud Marquart, Willem-Hendrik Gispen, and Pierre N. E. De Graan

Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, Utrecht University, Utrecht, The Netherlands

Abstract: The nervous tissue-specific protein B-50 (GAP-43), which has been implicated in the regulation of neurotransmitter release, is a member of a family of atypical calmodulin-binding proteins. To investigate to what extent calmodulin and the interaction between B-50 and calmodulin are involved in the mechanism of Ca^{2+}-induced noradrenaline release, we introduced polyclonal anti-calmodulin antibodies, calmodulin, and the calmodulin antagonists trifluoperazine, W-7, calmidazolium, and polymyxin B into streptolysin-O-permeated synaptosomes prepared from rat cerebral cortex. Anti-calmodulin antibodies, which inhibited Ca^{2+}/calmodulin-dependent protein kinase II autophosphorylation and calcineurin phosphatase activity, decreased Ca^{2+}-induced noradrenaline release from permeated synaptosomes. Exogenous calmodulin failed to modulate release, indicating that if calmodulin is required for vesicle fusion it is still present in sufficient amounts in permeated synaptosomes. Although trifluoperazine, W-7, and calmidazolium inhibited Ca^{2+}-induced release, they also strongly increased basal release. Polymyxin B potently inhibited Ca^{2+}-induced noradrenaline release without affecting basal release. It is interesting that polymyxin B was also the only antagonist affecting the interaction between B-50 and calmodulin, thus lending further support to the hypothesis that B-50 serves as a local Ca^{2+}-sensitive calmodulin store underneath the plasma membrane in the mechanism of neurotransmitter release. We conclude that calmodulin plays an important role in vesicular noradrenaline release, probably by activating Ca^{2+}/calmodulin-dependent enzymes involved in the regulation of one or more steps in the release mechanism. **Key Words:** Calmodulin—Calcium—Exocytosis—B-50/GAP-43—Calmodulin antagonists—Permeated synaptosomes.
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B-50 (also known as GAP-43, neuromodulin, and F1) is a neuronal member of a family of protein kinase C (PKC) substrates that share a common amino acid sequence (referred to as the IQ domain) possessing atypical calmodulin (CaM)-binding properties (Alexander et al., 1987). Other members of this family are RC3/neurogranin, p68 RNA helicase, and Igloo (Buelt et al., 1994; Gerendasy et al., 1995; and references therein). Both in purified systems (Liu and Storm, 1990) and in synaptosomal plasma membranes (SPMs) (De Graan et al., 1990), B-50 binds CaM in the absence of Ca^{2+} and dissociates from CaM in the presence of Ca^{2+}. PKC-mediated phosphorylation of Ser^{41} within the presumed CaM-binding domain of rat B-50 (residues 39–51) blocks the interaction between B-50 and CaM, and vice versa, CaM binding blocks B-50 phosphorylation by PKC, indicating that B-50 phosphorylation and B-50/CaM binding are mutually exclusive (Alexander et al., 1987; De Graan et al., 1990; Chapman et al., 1991). At present, a large body of evidence exists implicating B-50 in synaptic plasticity processes in the brain, including neurite outgrowth, long-term potentiation, and neurotransmitter release (reviewed by Liu and Storm, 1990; Strittmatter et al., 1992; De Graan and Gispen, 1993). The involvement of the presynaptic membrane-associated protein B-50 in Ca^{2+}-induced neurotransmitter release was demonstrated by the introduction of anti-B-50 antibodies (IgGs) in synaptosomes (Dekker et al., 1989, 1991; Hens et al., 1993*a*) and PC12 cells (Norden et al., 1991) and by the expression of antisense B-50 mRNA in PC12 cells (Ivins et al., 1993). Although the exact

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Address correspondence and reprint requests to Dr. P. N. E. De Graan at Department of Medical Pharmacology, Rudolf Magnus Institute for Neurosciences, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.

Abbreviations used: CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CMZ, calmidazolium; DSS, disuccinimidyl suberate; NA, noradrenaline; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMB, polymyxin B; SDS, sodium dodecyl sulfate; SLO, streptolysin-O; SPM, synaptosomal plasma membrane; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

role of B-50 in neurotransmitter release has not been resolved yet, introduction of site-specific anti-B-50 IgGs and specific PKC inhibitors into streptolysin-O (SLO)-permeated synaptosomes convincingly demonstrated that the CaM-binding domain of B-50 plays an essential role after the Ca^{2+} trigger in this mechanism (Hens et al., 1993*b*, 1995).

The atypical CaM-binding properties of B-50 prompted several groups to suggest that B-50 serves as a local CaM store at the presynaptic plasma membrane, releasing CaM in response to a depolarization-induced Ca^{2+} influx or to receptor-mediated PKC activation and subsequent B-50 phosphorylation (Alexander et al., 1987; De Graan et al., 1990; Spencer and Willard, 1992). Once dissociated, CaM might regulate the activity of Ca^{2+}-dependent proteins and enzymes that are implicated in exocytosis, such as α-fodrin, Ca^{2+}/CaM-dependent protein kinase II (CaMKII), and Ca^{2+}/CaM-dependent protein phosphatase 2B, also known as calcineurin. Anti-α-fodrin IgGs, for instance, have been shown to reduce catecholamine release from permeated chromaffin cells (Perrin et al., 1987). Introduction of permanently activated CaMKII into squid giant synapses (Llinás et al., 1985) and brain synaptosomes (Nichols et al., 1990) potentiated vesicular neurotransmitter release, most likely through the phosphorylation of synapsin I, which links transmitter vesicles to actin filaments (reviewed by Greengard et al., 1993). In permeated *Paramecium* cells (Momayezi et al., 1987) and brain synaptosomes (De Graan et al., 1992) anti-calcineurin IgGs inhibited Ca^{2+}-induced exocytosis, indicating that Ca^{2+}/CaM-dependent protein dephosphorylation is important in Ca^{2+}-induced release.

The use of CaM antibodies and antagonists has provided more direct evidence for a role of CaM in Ca^{2+}-induced neurotransmitter release from nerve terminals, although conclusive evidence is still lacking, because (a) CaM antagonists that inhibited secretion from intact and permeated cells have limited specificity (Hidaka et al., 1981; Schatzman et al., 1983) and (b) a reduction in secretion from intact cells after manipulation of CaM activity using either CaM antagonists (Sasakawa et al., 1983; Clapham and Neher, 1984; Sitges and Talamo, 1993) or intracellular application of anti-CaM IgGs (Kenigsberg and Trifaró, 1985) was partially due to inhibition of the Ca^{2+} entry (reviewed by Augustine et al., 1987; Trifaró et al., 1992). This latter problem can be avoided by the use of Ca^{2+} ionophores or cell permeation techniques, and, indeed, using the former approach, an inhibition of secretion that could not be attributed to a block of Ca^{2+} entry was reported for the CaM antagonist trifluoperazine (TFP) in chromaffin cells (Kenigsberg et al., 1982; Clapham and Neher, 1984).

To investigate to what extent CaM and its interaction with B-50 are important in the mechanism of vesicular neurotransmitter release, we tested the effects of polyclonal anti-CaM IgGs, exogenous CaM, and several CaM antagonists on Ca^{2+}-induced noradrenaline (NA) release from SLO-permeated synaptosomes. Our data provide evidence for a role of CaM in the molecular mechanism of vesicular NA release and substantiate a role of B-50 as a local Ca^{2+}-sensitive CaM store underneath the presynaptic plasma membrane in this mechanism.

MATERIALS AND METHODS

Materials

Brains of male Wistar rats weighing 100–120 g were used. SPMs were prepared from rat forebrain as described (Kristjansson et al., 1982). Synaptosomes from rat cerebral cortex were isolated on Percoll-sucrose gradients (15–23% interface) as described (Dunkley et al., 1988). Rat B-50 was purified according to a previously described method (Zwiers et al., 1985), followed by affinity chromatography on CaM-agarose (Andreasen et al., 1983). L-[7,8-^{3}H]NA (34 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) were purchased from Amersham (Buckinghamshire, U.K.), and ^{125}I-Bolton–Hunter-labeled CaM (82.5 μCi/μg of protein) was from NEN (Dreieich, Germany). TFP was obtained from Fluka (Buchs, Switzerland). Bacitracin, calcineurin (4,000 IU/mg of protein), CaM (65,000 IU/mg of protein), CaM-agarose, calmidazolium (CMZ), polymyxin B (PMB; 7,800 IU/mg of protein), and W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] were from Sigma. All other chemicals were from Merck (Darmstadt, Germany). CMZ was dissolved as a 50 m*M* stock solution in dimethyl sulfoxide.

Preparation of anti-CaM IgGs

Polyclonal anti-CaM serum 8917 was obtained by multiple intradermal injections of White New Zealand rabbits with bovine CaM conjugated to keyhole limpet hemocyanin (Goodall et al., 1983), using 1 mg of conjugate in Freund's complete adjuvant for priming and 1 mg of conjugate in Freund's incomplete adjuvant for boosters 3 and 6 weeks later. Anti-CaM IgGs were purified from antiserum 8917 by affinity chromatography on a bovine CaM-agarose column as described (Oestreicher et al., 1983), dialyzed extensively against 1,000 volumes of 124 m*M* NaCl and 5 m*M* PIPES (pH 6.8), and stored at −80°C at a concentration of 0.33 mg of IgG/ml. Total rabbit IgGs (ICN, Zoetermeer, the Netherlands) were used as a control.

Western blotting

Protein samples in denaturation buffer [final concentrations, 62.5 m*M* Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue, pH 6.5] were separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically blotted onto nitrocellulose. Blots were fixed using 2.5% glutaraldehyde and 4% formaldehyde and blocked with 10% skimmed milk proteins before immunostaining with anti-CaM 8917 IgGs (diluted 1:100) in Tris-buffered saline containing 0.5% Tween-20 (pH 7.4).

Protein phosphorylation

Aliquots of SPMs (10 μg of protein in a final volume of 25 μl) were preincubated with exogenous CaM (5 IU) and IgGs for 5 min at 30°C in buffer A (10 m*M* Tris-HCl, 10 m*M* $MgCl_2$, and 0.1 m*M* $CaCl_2$, pH 7.4). Phosphorylation was performed with [γ-^{32}P]ATP (7.5 μ*M*, 1 μCi) for 15 s as described previously (Hens et al., 1993*a*).

B-50 dephosphorylation

Purified B-50 was phosphorylated with [γ-^{32}P]ATP by PKC before dephosphorylation as described previously (Hens et al., 1995). Calcineurin (0.5 IU) in the presence of CaM (5 IU) was used to dephosphorylate aliquots of ^{32}P-B-50 (0.02 μg of protein in a final volume of 20 μl) in buffer A supplemented with 0.2 mg/ml of bacitracin for 30 min at 30°C. Anti-CaM IgGs, CaM, and calcineurin were added simultaneously to ^{32}P-B-50. Proteins were separated by 11% SDS-PAGE, and B-50 phosphorylation was quantified by densitometry of the autoradiogram.

NA release from SLO-permeated synaptosomes

Synaptosomes (10 μg of protein in a final volume of 60 μl), labeled with [^{3}H]NA, were permeated with 0.3 IU/ml of SLO (Murex, Utrecht, The Netherlands) in the presence of 2 m*M* ATP and either 10^{-8} or 10^{-5} *M* free Ca^{2+} (generated by Ca^{2+}/EGTA buffers containing a final concentration of 10 m*M* EGTA) for 5 min at 25°C as described earlier (Hens et al., 1995). Ca^{2+}-induced [^{3}H]NA release, expressed as a percentage of total synaptosomal [^{3}H]NA, was calculated by subtracting the percentage of NA release in the presence of 10^{-8} *M* Ca^{2+} from that released in the presence of 10^{-5} *M* Ca^{2+}.

B-50/CaM cross-linking

B-50 and CaM were cross-linked using disuccinimidyl suberate (DSS; Pierce, Oud Beijerland, The Netherlands) as described earlier (De Graan et al., 1990; Hens et al., 1995). In brief, purified B-50 (0.15 μg of protein in a final volume of 26 μl) was incubated with 13 IU of CaM in buffer B (20 m*M* PIPES, 10 m*M* $MgCl_2$, and 5 m*M* EGTA, pH 7.4) for 10 min at 37°C, whereas synaptosomes (10 μg of protein in a final volume of 60 μl) were permeated as described above in the presence of 30 IU of CaM and the indicated [Ca^{2+}] for 2.5 min at 37°C. Thereafter DSS was added at a final concentration of 1 m*M*, and the incubation was continued for 10–15 min at 37°C. The indicated amounts of PMB or W-7 were present throughout the experiment. Proteins in denaturation buffer were separated by SDS-PAGE and western-blotted. B-50/CaM complex formation was analyzed densitometrically after B-50 immunostaining.

CaM-agarose affinity chromatography of B-50

Aliquots (50 μg of protein) of a semipurified preparation of rat B-50 that was obtained by a previously described method (De Graan et al., 1993) were incubated with 1 ml of CaM-agarose (1 mg of CaM/ml) in buffer C [20 m*M* HEPES, 1 m*M* EGTA, 1 m*M* dithiothreitol, and 0.1% (vol/vol) Triton X-100, pH 7.4] for 1 h at 18°C. The CaM-agarose was spun down (5 min, 10,000 *g*) and subsequently washed two or three times in 1 ml of buffer C (30 min at 18°C) to remove unbound proteins. Next, B-50 elution from CaM-agarose was established in the discarded supernatant (5 min, 10,000 *g*) of three or four subsequent washes (30 min at 18°C) in 1 ml of either buffer C with PMB or buffer D [20 m*M* HEPES, 3 m*M* $CaCl_2$, 1 m*M* dithiothreitol, and 0.1% (vol/vol) Triton X-100, pH 7.4]. Aliquots (20 μl) of discarded supernatant in denaturation buffer were separated by 11% SDS-PAGE. B-50 was detected by protein silver staining according to the method described by Merril et al. (1981).

Other determinations

Protein content was measured by the technique of Bradford (1976) using bovine serum albumin as the standard.

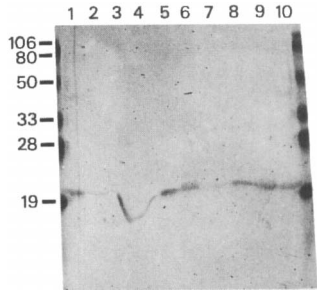

FIG. 1. Identification of CaM by anti-CaM 8917 IgGs on western blots loaded with CaM, SPMs, synaptosomes and brain homogenates. Bovine CaM (0.5 μg of protein; lanes 1, 3, and 10), rat CaM (0.5 μg of protein; lane 9), rat SPMs (25 μg of protein; lane 8), synaptosomes from rat cerebral cortex (25 μg of protein; lanes 2, 4, 7), and homogenates (25 μg of protein) of rat hippocampus (lane 5) and of rat total forebrain (lane 6) were separated by SDS-PAGE (15% gels), blotted, fixed, and immunostained with affinity-purified rabbit anti-bovine CaM 8917 IgGs (diluted 1:100). EGTA was present at a final concentration of 2.5 m*M* in all samples, except for the samples in lanes 3 and 4, which contained 2 m*M* Ca^{2+}. (Note the migration shift of CaM.) Molecular weight markers are indicated on the left.

Data are mean ± SEM values, with n being the number of independent experiments. Statistical analysis was performed with a two-tailed Student's *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of anti-CaM IgGs on NA release

To study the role of CaM in the mechanism of Ca^{2+}-induced NA release we introduced anti-CaM IgGs into the interior of SLO-permeated synaptosomes. Therefore, we raised a rabbit anti-bovine CaM antiserum (coded 8917), affinity-purified its IgGs on a bovine CaM-agarose column, and tested the specificity of the IgG fraction by immunostaining of western blots (Fig. 1). Affinity-purified 8917 IgGs reacted with bovine CaM as well as with rat CaM (Fig. 1, lanes 9 and 10), as was expected from the almost identical amino acid sequence of CaM in both species (Wylie and Vanaman, 1988). A single band that comigrated with purified CaM was also specifically recognized by 8917 IgGs in rat total forebrain and hippocampus homogenates, in highly purified synaptosomes from rat cerebral cortex, and in rat SPMs (Fig. 1). The characteristic, Ca^{2+}-dependent migration shift of CaM on SDS-PAGE (Fig. 1, lanes 1–4) indicated that 8917 IgGs recognized CaM in its Ca^{2+}-bound as well as in its Ca^{2+}-depleted form. In addition, 10 μl of 8917 IgGs was able to immunoprecipitate ^{125}I-CaM [0.2 μg of protein, 22.5 nCi in a final volume of 400 μl as described earlier (De Graan et al., 1989; data not shown)], further demonstrating the specificity of 8917 IgGs toward CaM. Thus, our data show that 8917 IgGs specifically recognize CaM in solution (see also Figs. 2 and 3) as well as bound to a solid phase [nitrocellulose (Fig. 1) or ELISA plates (data not shown)].

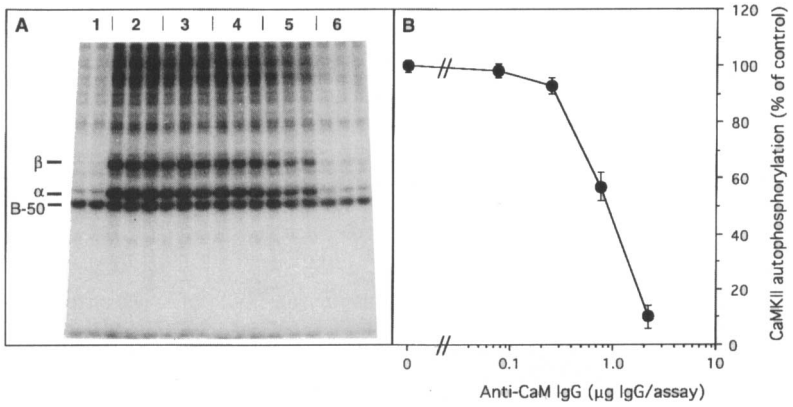

FIG. 2. Effects of anti-CaM IgGs on Ca^{2+}/CaM-dependent protein phosphorylation in SPMs. **A:** Representative autoradiogram of SPMs (10 μg of protein) phosphorylated with [γ-^{32}P]ATP (1 μCi; 7.5 μM) in the absence (lane 1) or presence (lanes 2–6; 5 IU) of CaM. Anti-CaM 8917 IgGs were either absent (lanes 1 and 2) or present at 0.075 (lane 3), 0.25 (lane 4), 0.75 (lane 5), or 2.25 (lane 6) μg of IgG/25-μl assay mixture throughout the 5-min preincubation and 15-s phosphorylation experiment. B-50 and the α and β subunit of CaMKII are indicated. **B:** Inhibition of the autophosphorylation of the 60-kDa β subunit of CaMKII by anti-CaM 8917 IgGs. Protein phosphorylation was quantified by densitometry of autoradiograms and expressed as a percentage of controls without IgGs. Data are mean ± SEM (bars) values of 10 observations obtained from four independent experiments.

Subsequently, anti-CaM 8917 IgGs were tested for their ability to interfere with two CaM-dependent processes: CaMKII autophosphorylation in SPMs (Fig. 2) and calcineurin-mediated B-50 dephosphorylation (Fig. 3). Autophosphorylation of the 50-kDa α- and 60-kDa β-subunits of CaMKII (reviewed by Colbran and Soderling, 1990), which was only visible in SPMs when exogenous CaM was added (Fig. 2A), was inhibited by anti-CaM IgGs in a concentration-dependent manner (Fig. 2B). Inhibition of CaMKII autophosphorylation was half-maximal at ~0.75 μg of anti-CaM IgG per assay ($p < 0.001$) and almost complete (90%) at 2.25 μg of anti-CaM IgG per assay. Control, total rabbit IgGs were without effect on CaMKII autophosphorylation. Phosphorylation of PKC substrate B-50 was not affected by anti-CaM IgGs (Fig. 2A).

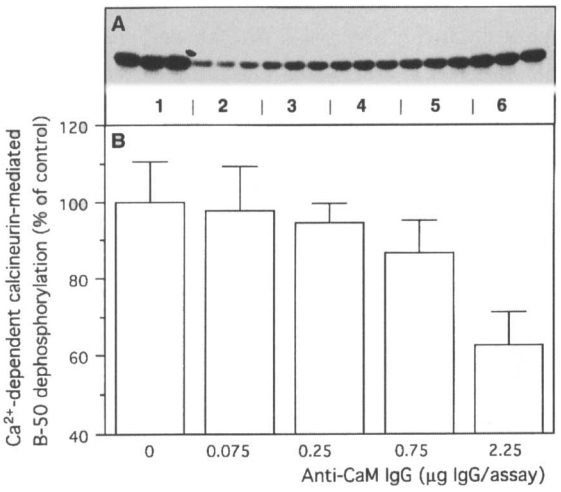

FIG. 3. Effect of anti-CaM IgGs on calcineurin-mediated B-50 dephosphorylation. Calcineurin was used to dephosphorylate purified, PKC-phosphorylated ^{32}P-B-50 for 30 min at 30°C in the presence of increasing concentrations of anti-CaM 8917 IgGs. **A:** Autoradiogram of ^{32}P-B-50 before (lane 1) and after (lanes 2–6) dephosphorylation in the absence (lanes 1, 2, and 6) or presence of 0.75 (lane 3), 1.5 (lane 4), or 2.25 (lane 5) μg of anti-CaM 8917 IgGs/20-μl assay mixture. Ca^{2+}-independent B-50 dephosphorylation in the presence of 5 mM EGTA is shown in lane 6 and represented <30% of the total B-50 dephosphorylation. **B:** Effect of anti-CaM IgGs on Ca^{2+}-dependent B-50 dephosphorylation by calcineurin. B-50 dephosphorylation was significantly inhibited at 2.25 μg of anti-CaM IgG per assay ($p < 0.05$). Data are mean ± SEM (bars) values of nine observations obtained from three independent experiments.

PKC-phosphorylated ^{32}P-B-50 was used to study the effect of anti-CaM IgGs on calcineurin phosphatase activity (Fig. 3). Calcineurin dephosphorylated ~50% of ^{32}P-B-50 (20 ng of protein) in 30 min. More than 70% of this B-50 dephosphorylation occurred in a Ca^{2+}/CaM-dependent manner (Fig. 3A). These data are in accordance with previous reports that calcineurin dephosphorylated B-50 in vitro in a mainly Ca^{2+}/CaM-dependent manner [>60%, depending of the quality of the calcineurin preparation and the assay conditions (see also Pallen and Wang, 1984)] (Liu and Storm, 1989; Schrama et al., 1989). Anti-CaM IgGs inhibited Ca^{2+}/CaM-dependent B-50 dephosphorylation by calcineurin in a concentration-dependent manner. A maximal inhibition of ~40% was observed in the presence of 2.25 μg of anti-CaM IgG per assay ($p < 0.05$), the highest concentration tested (Fig. 3B). Control, total rabbit IgGs were without effect on dephosphorylation.

The effect of anti-CaM IgGs on NA release was tested in SLO-permeated synaptosomes (Fig. 4). Under optimal conditions for SLO permeation, an immediate increase in the extracellular free [Ca^{2+}] from 10^{-8} to 10^{-5} M triggered the release of NA-containing vesicles from permeated synaptosomes, constituting 11.7 ± 0.5% of the total synaptosomal [^{3}H]NA content, which was referred to as Ca^{2+}-induced NA release (see also Hens et al., 1993*a*, 1995). Introduction of anti-CaM 8917 IgGs into SLO-permeated synaptosomes decreased Ca^{2+}-induced NA release in a concentration-dependent manner: 32% in the presence of 10 μg of anti-CaM IgG per assay ($p < 0.001$), the highest concentration of anti-CaM IgGs tested, and 23% in the presence of 5 μg of anti-CaM IgG per assay ($p < 0.02$). Ca^{2+}-induced NA release decreased

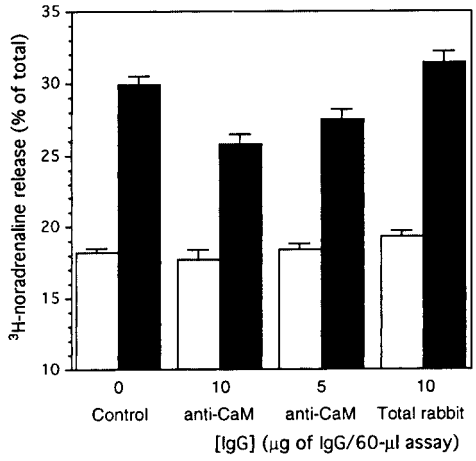

FIG. 4. Effect of anti-CaM IgGs on Ca^{2+}-induced [^{3}H]NA release from SLO-permeated synaptosomes. Synaptosomes were permeated in the presence of 10^{-8} (□) or 10^{-5} *M* (■) Ca^{2+} for 5 min at 25°C. Anti-CaM 8917 IgGs or control, total rabbit IgGs were present at the indicated amounts in a final volume of 60 μl throughout the experiment. Data are mean ± SEM (bars) values of six to 12 observations obtained from three independent experiments.

to 8.0 ± 0.8 or 9.0 ± 0.7% in the presence of 10 or 5 μg of anti-CaM IgG per assay, respectively. Basal NA release (in the presence of 10^{-8} *M* Ca^{2+}) was not affected by anti-CaM IgGs. Control, total rabbit IgGs (10 μg of IgG per assay) were without effect on Ca^{2+}-induced NA release (Fig. 4).

Previously, cross-linker DSS was used to demonstrate that CaM and B-50 interact in the absence of Ca^{2+} (De Graan et al., 1990; Hens et al., 1995). DSS treatment of purified proteins or SPMs resulted in the formation of 70-kDa B-50/CaM complexes (1:1 stoichiometry), which were clearly visible on western blots after immunostaining for B-50 or CaM. Previously, we have demonstrated that monoclonal anti-B-50 NM2 IgGs, which blocked Ca^{2+}-induced NA release, inhibited the interaction between B-50 and CaM observed after DSS treatment (Hens et al., 1995). Here we used this cross-linker approach to study the effect of anti-CaM IgGs on the B-50/CaM interaction. However, our attempts with polyclonal anti-CaM IgGs failed, because in the presence of DSS high-molecular-weight complexes were formed, consisting of multiple molecules of B-50, CaM, and IgG, which did not enter the stacking gel (5% SDS-PAGE) during gel electrophoresis (data not shown). Similar complexes were not observed in the presence of anti-B-50 NM2 IgGs (Hens et al., 1995), probably because these monoclonal IgGs and CaM bound competitively to B-50.

Effects of exogenous CaM on NA release

To manipulate endogenous synaptosomal CaM levels we introduced CaM into the interior of SLO-permeated synaptosomes and tested the effects on Ca^{2+}-induced NA release. Permeation of synaptosomes in the presence of ≤6 μ*M* of bovine CaM affected neither basal NA release nor Ca^{2+}-induced NA release (data not shown), suggesting that if CaM is required for the induction of NA release it is still present in sufficient amounts in SLO-permeated synaptosomes. A second possibility is that CaM is required for sustained NA release from permeated synaptosomes. As has been shown for the secretory responsiveness in other permeation systems (reviewed by Morgan et al., 1993), the Ca^{2+}-induced NA release response of synaptosomes decreased in a time-dependent manner when the Ca^{2+} trigger was applied after prolonged periods of permeation and was completely lost after 10 min of permeation in the presence of 0.3 IU/ml of SLO at 25°C (J.J.H.H., unpublished data). Incubation of SLO-permeated synaptosomes with 0.5–5.0 μ*M* CaM (in either 10^{-8} or 10^{-6} *M* Ca^{2+} buffered with 1 m*M* EGTA) for 2.5 min at 25 or 37°C before application of the Ca^{2+} trigger (10^{-5} *M* Ca^{2+} buffered with 10 m*M* EGTA) neither restored the Ca^{2+}-induced NA release response nor prevented its progressive decline (data not shown).

Effects of CaM antagonists on NA release

The CaM antagonists TFP, W-7, and CMZ, which inhibit CaM only in the presence of Ca^{2+} (Tanaka and Hidaka, 1980), were tested for their effect on Ca^{2+}-induced NA release from permeated synaptosomes (Fig. 5A). At 10 μ*M* TFP and CMZ decreased Ca^{2+}-induced NA release significantly by 32 and 23%, respectively. Ca^{2+}-induced NA release decreased from 12.0 ± 0.8 (control) to 8.2 ± 1.7% in the presence of TFP and to 9.2 ± 1.2% in the presence of CMZ. At 100 μ*M* TFP, W-7, or CMZ, Ca^{2+}-induced NA release was completely inhibited. At this concentration TFP, W-7, and CMZ increased basal NA release from 17 to 42, 31, and 72%, respectively (Fig. 5A). PMB, known as a potent inhibitor of neurotransmitter release from intact (Dekker et al., 1990) and permeated (Dekker et al., 1991; Hens et al., 1993*a*,*b*) synaptosomes, inhibited Ca^{2+}-induced release by 75% at 200 IU/ml in these experiments (Fig. 5A). This potent inhibitory effect of PMB prompted us to characterize its effect on NA release more extensively. We observed that PMB inhibited Ca^{2+}-induced NA release half-maximally at ~50 IU/ml (Fig. 5B). This PMB-mediated inhibition of Ca^{2+}-induced NA release was not due to a reduction in the SLO permeation rate of the synaptosomal preparation, as was established by measuring the synaptosomal efflux of the cytosolic marker protein lactate dehydrogenase (data not shown; see also Hens et al., 1993*a*).

To establish whether CaM and B-50 interact in nerve terminals, we treated SLO-permeated synaptosomes with the cross-linker DSS (Fig. 6). As observed in previous studies on purified proteins and SPMs (De Graan et al., 1990; Hens et al., 1995), DSS treatment of SLO-permeated synaptosomes also resulted in the formation of B-50/CaM complexes, which were clearly visible as a single 70-kDa band on western

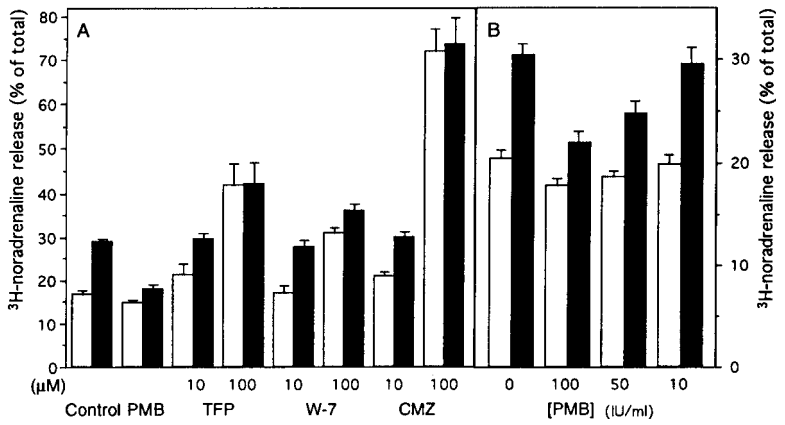

FIG. 5. Effects of antagonists TFP, W-7, CMZ, and PMB on Ca^{2+}-induced [^{3}H]NA release from SLO-permeated synaptosomes. Synaptosomes were permeated in the presence of 10^{-8} (□) or 10^{-5} *M* (■) Ca^{2+}. Antagonists were present throughout the experiment. NA release was determined in the supernatant after incubation for 5 min at 25°C. **A:** Effects of Ca^{2+}/CaM antagonists TFP, W-7, and CMZ. PMB (200 IU/ml, ~20 μ*M*) was used as a positive control. Secretion from intact, control synaptosomes was 10.7 ± 0.8% of the total [^{3}H]NA content in these experiments. Data are mean ± SEM (bars) values of nine to 18 observations obtained from three independent experiments. **B:** Concentration dependency of the effect of PMB on Ca^{2+}-induced [^{3}H]NA release from SLO-permeated synaptosomes (10 IU/ml PMB is ~1 μ*M*). Data are mean ± SEM (bars) values of six to 18 observations obtained from three independent experiments. Note the difference in scaling of the *y*-axes of A and B.

blots after immunostaining for B-50 (data not shown). Most B-50/CaM complexes were formed in the presence of 10^{-8} or 10^{-7} *M* Ca^{2+} in SLO-permeated synaptosomes. The B-50/CaM complex dissociated in a Ca^{2+}-dependent manner; dissociation was significant at a [Ca^{2+}] of $>3 \times 10^{-7}$ *M* and half-maximal at $\sim 3 \times 10^{-6}$ *M* Ca^{2+} (Fig. 6).

These data prompted us to study the effect of PMB on B-50/CaM binding in the absence of Ca^{2+} (Fig. 7). PMB inhibited B-50/CaM complex formation in a concentration-dependent manner (half-maximal dissociation at 400 IU/ml of PMB), whereas W-7 at 100 μ*M* was without effect (Fig. 7A). To exclude the possibility that the inhibition of B-50/CaM binding was due to an effect of PMB on DSS-mediated cross-linking, we also tested the effect of PMB on B-50/CaM binding by CaM-agarose affinity chromatography of B-50 (Fig. 7B–D). From a semipurified preparation of rat B-50 (Fig. 7B–D, lanes 1), which contained intact B-50 as well as a 40-kDa proteolytic B-50 fragment, only intact B-50 bound to CaM-agarose in the presence of EGTA and dissociated from CaM-agarose in the presence of Ca^{2+} (Fig. 7B). CaM-agarose that was incubated with B-50 for 1 h at 18°C did not release any detectable B-50 after a third wash in EGTA-containing buffer C (Fig. 7B–D, lanes 3), indicating that B-50 was firmly attached to CaM-agarose. In the presence of 400 IU/ml of PMB B-50 started to dissociate from CaM-agarose (four washes in EGTA-containing buffer C), and at 4,000 IU/ml of PMB all attached B-50 was removed from CaM-agarose within four washes (Fig. 7C). PMB at 40,000 IU/ml dissociated all attached B-50 from CaM-agarose in only two washes in EGTA-containing buffer C (Fig. 7D). Subsequent washes in Ca^{2+}-containing buffer D did not dissociate any more B-50 from CaM-agarose. These data confirm that PMB dissociated the B-50/CaM complex in a concentration-dependent manner.

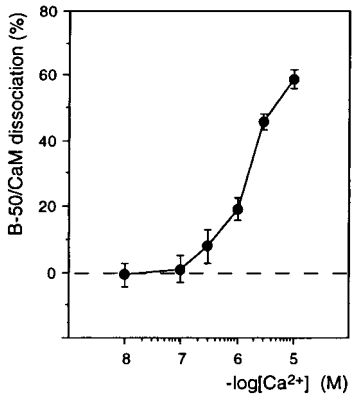

FIG. 6. Ca^{2+} sensitivity of B-50/CaM dissociation in SLO-permeated synaptosomes. Synaptosomes were permeated with 0.3 IU/ml of SLO at the indicated free Ca^{2+} concentrations for 2.5 min at 37°C before cross-linker DSS was added for another 10 min. B-50/CaM complex formation was analyzed as described in Materials and Methods and expressed as a percentage of the value with 10^{-8} *M* Ca^{2+} (100%). B-50/CaM dissociation was calculated by subtracting the percentage of B-50/CaM complex formation from 100%. Data are mean ± SEM (bars) values of nine observations obtained from three independent experiments.

DISCUSSION

CaM and Ca^{2+}-induced NA release

A role of CaM in Ca^{2+}-induced exocytosis of neurotransmitters has not yet been established conclusively and is an important matter of debate, as synaptosomal catecholamine release in mice lacking the CaMKII substrate synapsin I was found to be unaffected (Rosahl et al., 1993). In the present study we used the SLO-permeated synaptosome preparation, a well-characterized system to study the molecular mechanism of vesicular neurotransmitter release (reviewed by De Graan and Gispen, 1993), to investigate the role of CaM in the later stages of the Ca^{2+}-induced release process. The main finding of this study is that anti-CaM IgGs, which were characterized for their speci-

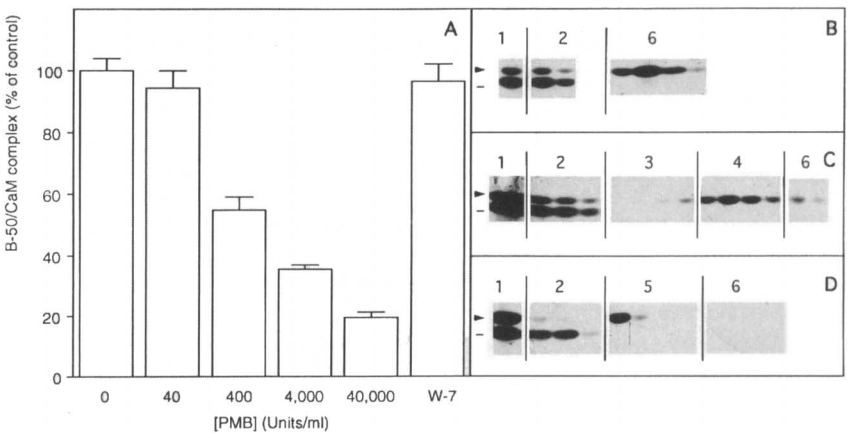

FIG. 7. Effect of PMB on the interaction between B-50 and CaM. **A:** Purified B-50 and CaM were cross-linked with DSS in the presence of 5 m*M* EGTA ($[Ca^{2+}]_{free}$ < 10^{-8} *M*) and either 10^{-4} *M* W-7 or increasing concentrations of PMB (40 IU/ml of PMB is ~4 μM) for 15 min at 37°C. B-50/CaM complex formation was analyzed as described in Materials and Methods and expressed as a percentage of untreated controls. Data are mean ± SEM (bars) values of two to eight observations obtained from four independent experiments. **B–D:** CaM-agarose affinity chromatography of B-50 represented by protein silver stainings of 11% SDS-PAGE gels of single, representative experiments. A semipurified preparation of rat B-50 (lanes 1) was incubated with CaM-agarose in 1 m*M* EGTA-containing buffer C for 1 h at 18°C, after which excess proteins were removed by two or three subsequent washes as described in Materials and Methods (lanes 2). Note the presence of a 40-kDa proteolytic fragment of B-50 (McMaster et al., 1988) in these washes (arrowheads indicate intact B-50; lines indicate the 40-kDa fragment of B-50). Next CaM-agarose was incubated three or four subsequent times with 400 (C; lanes 3), 4,000 (C; lanes 4), or 40,000 (D; lanes 5) IU/ml of PMB. At the end of each experiment any remaining B-50 was removed from CaM-agarose by two to four subsequent incubations in 3 m*M* Ca^{2+} (B–D; lanes 6). Lanes were loaded with 20-μl aliquots of discarded supernatants.

ficity against CaM and ability to interfere functionally with Ca^{2+}/CaM-dependent enzyme activities, decreased Ca^{2+}-induced NA release from SLO-permeated synaptosomes.

The polyclonal IgGs recognized only CaM in, e.g., synaptosomes and SPMs prepared from rat cerebral cortex (Fig. 1), thus demonstrating their specificity and the presence of endogenous CaM in these preparations. The ability of the anti-CaM IgGs to interfere with Ca^{2+}/CaM-dependent enzyme activities (Figs. 2 and 3) shows their suitability for physiological interference studies in SLO-permeated synaptosomes. Introduction of anti-CaM IgGs into permeated synaptosomes decreased Ca^{2+}-induced NA release in a concentration-dependent manner. Maximal inhibition of the Ca^{2+}-induced vesicular NA release by anti-CaM IgGs was 32%. Higher concentrations of anti-CaM IgGs could not be tested. Basal NA release was unaffected by the IgGs. Thus, anti-CaM IgGs inhibit Ca^{2+}-induced NA release from permeated synaptosomes only partially. In earlier studies it was also shown that introduction of anti-CaM IgGs into chromaffin cells (Kenigsberg and Trifaró, 1985) and PC12 cells (Ahnert-Hilger et al., 1989) partially inhibited Ca^{2+}-induced neurotransmitter release. Because CaM interacts with several different proteins under different conditions and with different affinities (Klee, 1988; O'Neil and DeGrado, 1990), it is possible that distinct CaM-sensitive steps are differentially affected by the anti-CaM IgGs. Indeed, we observed that anti-CaM IgGs blocked CaMKII autophosphorylation almost completely (90%), whereas Ca^{2+}/CaM-dependent B-50 dephosphorylation by calcineurin was decreased only partially (40%). Therefore, the most likely interpretation of our data is that the molecular mechanism of neurotransmitter release is only partially CaM dependent, e.g., only the process of vesicle recruitment or vesicle recycling, and that other molecular events are CaM independent, e.g., vesicle fusion, and involve other Ca^{2+}-binding proteins, such as, e.g., synaptotagmin (Brose et al., 1992).

Although anti-CaM IgGs decreased Ca^{2+}-induced NA release, introduction of exogenous CaM into permeated synaptosomes did not affect Ca^{2+}-induced NA release, indicating that CaM is still present in sufficient amounts in permeated synaptosomes. These data are in line with earlier studies reporting that CaM addition failed to affect Ca^{2+}-induced exocytosis from permeated chromaffin cells (Sarafian et al., 1987), although CaM, along with other proteins, was partially lost from the cytosolic compartment after permeation (Vitale et al., 1992). Therefore, we assume that the intrasynaptosomal $[Ca^{2+}]$ determines the exchange of CaM between various CaM-binding proteins, e.g., CaMKII and calcineurin in the presence of Ca^{2+} and B-50 in the absence of Ca^{2+}, without inducing its release in the cytosol. Indeed, CaM was still present in SPMs that had been extensively washed on an alternating basis in 5 m*M* EGTA or 1 m*M* $CaCl_2$ (De Graan et al., 1990). A Ca^{2+}-dependent shuttling of CaM between membrane-associated, CaM-binding proteins may thus have largely prevented the loss of membrane-bound CaM after permeation. To study the involvement of this membrane-bound CaM in exocytosis in more detail, we used CaM antagonists.

PMB, a unique CaM antagonist inhibiting Ca^{2+}-induced NA release

Ca^{2+}-dependent CaM antagonists TFP, W-7, and CMZ have been used extensively to study the function of CaM in neurotransmitter release (reviewed by Augustine et al., 1987; Trifaró et al., 1992). In SLO-

permeated synaptosomes these antagonists indeed inhibited the Ca^{2+}-induced NA release. However, at high concentrations (100 μM) they also increased basal NA release. In permeated chromaffin cells a similar, large Ca^{2+}-independent catecholamine release was observed in the presence of TFP (>10 μM) and other CaM antagonists (Knight and Baker, 1982).

PMB was the only antagonist that completely inhibited Ca^{2+}-induced NA release in a concentration-dependent manner without increasing basal NA release (see also Dekker et al., 1991; Hens et al., 1993*a,b*). Earlier data had indicated that PMB inhibited CaM-dependent CaMKII autophosphorylation in SPMs more potently than PKC-mediated B-50 phosphorylation (Dekker et al., 1990), thus identifying PMB as a more potent antagonist of CaM than of PKC (Hegemann et al., 1991). Because PKC activation and PKC-mediated B-50 phosphorylation are not involved in the process of vesicular NA release from permeated synaptosomes after the Ca^{2+} trigger (Hens et al., 1993*a*), these data imply that the inhibition of Ca^{2+}-induced NA release by PMB is not due to an inhibition of PKC activity in this system. It is interesting that it appears that PMB interacts with CaM in a Ca^{2+}-independent fashion (Hegemann et al., 1991), an ability that PMB shares with anti-CaM IgGs and anti-B-50 NM2 IgGs, which also inhibit Ca^{2+}-induced NA release from permeated synaptosomes (Hens et al., 1995). PMB differs in this respect from the CaM antagonists TFP, W-7, and CMZ, which inhibit CaM only in the presence of Ca^{2+} (Tanaka and Hidaka, 1980). Accordingly, we observed that W-7 did not affect the interaction between B-50 and CaM, which is observed only in the absence of Ca^{2+} (De Graan et al., 1990; Liu and Storm, 1990), whereas PMB did (Fig. 7).

B-50 and Ca^{2+}-induced NA release

Introduction of monoclonal anti-B-50 NM2 IgGs into SLO-permeated synaptosomes has convincingly demonstrated that the CaM-binding domain of rat B-50 (amino acid residues 39–51) plays an important role in the process of Ca^{2+}-induced release of NA and neuropeptide cholecystokinin-8, presumably by serving as a local CaM store that is regulated in a Ca^{2+}- and phosphorylation-dependent fashion (Hens et al., 1993*b*, 1995). Indeed, we show in Fig. 6 that CaM dissociates from B-50 in SLO-permeated synaptosomes in a [Ca^{2+}]-dependent manner, which resembles the Ca^{2+} sensitivity of the vesicular release of NA and cholecystokinin-8 from this preparation (Hens et al., 1993*b*, 1995). It is tempting to speculate that anti-B-50 NM2 IgGs, anti-CaM IgGs, and PMB inhibit Ca^{2+}-induced NA release by affecting the molecular release machinery even before the release response is triggered by Ca^{2+}. In fact, anti-B-50 NM2 IgGs, PMB (Fig. 7), and probably also anti-CaM IgGs interfere with the interaction between B-50 and CaM in the absence of Ca^{2+}. This interference may result in the dissociation of CaM, thus depleting the local CaM store that is required for the Ca^{2+}-induced fusion and release of NA-containing vesicles.

In conclusion, we have presented evidence for an important role of CaM in the molecular mechanism of Ca^{2+}-induced vesicular NA release. Moreover, our data lend further support to the hypothesis that B-50 serves as a local, Ca^{2+}-sensitive CaM store underneath the presynaptic plasma membrane in the mechanism of catecholamine release from nerve terminals. The Ca^{2+} trigger dissociates CaM from B-50, and CaM subsequently activates one or more Ca^{2+}/CaM-dependent enzymes, which are implicated in the process of Ca^{2+}-induced neurotransmitter release, such as CaM-KII (Llinás et al., 1985; Nichols et al., 1990) and calcineurin (Momayezi et al., 1987; De Graan et al., 1992).

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