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Evidence for a Role of Protein Kinase C Substrate B-50 (GAP-43) in Ca^{2+}-Induced Neuropeptide Cholecystokinin-8 Release from Permeated Synaptosomes

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Abstract: To study the involvement of the protein kinase C (PKC) substrate B-50 [also known as growth-associated protein-43 (GAP-43), neuromodulin, and F1] in presynaptic cholecystokinin-8 (CCK-8) release, highly purified synaptosomes from rat cerebral cortex were permeated with the bacterial toxin streptolysin O (SL-O). CCK-8 release from permeated synaptosomes, determined quantitatively by radioimmunoassay, could be induced by Ca^{2+} in a concentration-dependent manner (EC_{50} of $\sim 10^{-5}$ *M*). Ca^{2+}-induced CCK-8 release was maximal at 10^{-4} *M* Ca^{2+}, amounting to ~10% of the initial 6,000 ± 550 fmol of CCK-8 content/mg of synaptosomal protein. Only 30% of the Ca^{2+}-induced CCK-8 release was dependent on the presence of exogenously added ATP. Two different monoclonal anti-B-50 antibodies were introduced into permeated synaptosomes to study their effect on Ca^{2+}-induced CCK-8 release. The N-terminally directed antibodies (NM2), which inhibited PKC-mediated B-50 phosphorylation, inhibited Ca^{2+}-induced CCK-8 release in a dose-dependent manner, whereas the C-terminally directed antibodies (NM6) affected neither B-50 phosphorylation nor CCK-8 release. The PKC inhibitors PKC_{19-36} and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), which inhibited B-50 phosphorylation in permeated synaptosomes, had no effect on Ca^{2+}-induced CCK-8 release. Our data strongly indicate that B-50 is involved in the mechanism of presynaptic CCK-8 release, at a step downstream of the Ca^{2+} trigger. As CCK-8 is stored in large dense-cored vesicles, we conclude that B-50 is an essential factor in the exocytosis from this type of neuropeptide-containing vesicle. The differential effects of the monoclonal antibodies indicate that this B-50 property is localized in the N-terminal region of the B-50 molecule, which contains the PKC phosphorylation site and calmodulin-binding domain. **Key Words:** Cholecystokinin-8—Neurotransmitter release—B-50/GAP-43—Calcium—Protein kinase C—Synaptosome.
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In the CNS, neuropeptides represent a large and diverse family of transmitters, which once released may act as local hormones inducing a wide variety of responses of longer duration (reviewed by Hökfelt, 1991). The neuropeptide cholecystokinin (CCK) is one of the most abundant endogenous peptidergic transmitters in the brain presently known (Rehfeld, 1985). Although structurally related peptides such as gastrins are only present in discrete brain regions, CCK occurs widely over most areas of the mammalian CNS, with highest concentrations in cerebral cortex (Marley et al., 1984). In rat cerebral cortex CCK is released predominantly as a sulfated octapeptide in a depolarization-induced and Ca^{2+}-dependent manner (Dodd et al., 1980; Emson et al., 1980; Verhage et al., 1991*a*). Besides its presence in cell bodies, CCK-8 is reported to be concentrated in synaptosomes, and CCK-8-like material has been demonstrated in synaptic vesicles (Pinget et al., 1978; Emson et al., 1980). CCK-8 is assumed to be released from large dense-cored vesicles by vesicle fusion outside the active zone (Golding and Bayraktaroglu, 1984; Zhu et al., 1986; Thureson-Klein and Klein, 1990; Verhage et al., 1991*b*) and is often colocalized with classical transmitters like dopamine (Hökfelt et al., 1980, 1987; Palacios et al., 1989) and amino acids (see Lopes da Silva et al., 1990) in the limbic system.

The trigger for CCK-8 release from large dense-cored vesicles, as for many other transmitters, is assumed to be $[Ca^{2+}]_i$ (Augustine et al., 1987; Smith and Augustine, 1988; Verhage et al., 1991*b*). The present knowledge about the molecular mechanism of release from this type of vesicle, however, is even more limited than the knowledge about release from small clear-cored vesicles (Südhof and Jahn, 1991). We

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Abbreviations used: CCK, cholecystokinin; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; NA, noradrenaline; PKC, protein kinase C; SL-O, streptolysin O.

have shown previously that polyclonal anti-B-50 antibodies, introduced into permeated synaptosomes, inhibited Ca^{2+}-induced noradrenaline (NA) release and B-50 phosphorylation, demonstrating that the nervous tissue-specific protein kinase C (PKC) substrate B-50 [also known as growth-associated protein-43 (GAP-43), neuromodulin, and F1 (for reviews, see Skene, 1989; Liu and Storm, 1990; Coggins and Zwiers, 1991; Gispen et al., 1991)] plays an important role in the mechanism of catecholamine release (Dekker et al., 1989, 1991*b*; Hens et al., 1993). Other studies have provided evidence that B-50 is involved in catecholamine release from PC12 cells (Norden et al., 1991; Ivins et al., 1993). B-50 has a single phosphorylation site for PKC at Ser^{41} (Coggins and Zwiers, 1989; Apel et al., 1990; Nielander et al., 1990), which is located within the proposed calmodulin-binding domain of B-50, extending from amino acid residue 39 to 51 (Chapman et al., 1991). Calmodulin binds to B-50 in the absence of Ca^{2+} and dissociates from B-50 in the presence of Ca^{2+} (De Graan et al., 1990; Liu and Storm, 1990) or after PKC-mediated B-50 phosphorylation (Alexander et al., 1987). Studies, using monoclonal antibodies directed against specific B-50 domains, suggest that the property of B-50 that is important in the mechanism of catecholamine release is located within the calmodulin-binding domain at the N-terminus of B-50 (De Graan et al., 1992; J. J. H. Hens et al., submitted for publication).

The involvement of B-50 in neurotransmitter release has only been demonstrated for catecholamines, which may be released from large dense-cored as well as from small clear-cored vesicles (Klein et al., 1982; Lundberg and Hökfelt, 1983; Zhu et al., 1986). Therefore, it remains to be established for which type of vesicle B-50 is involved in exocytosis. Because B-50 appears not to be colocalized with any particular neurotransmitter or type of vesicle (Benowitz et al., 1988), we focus in this article on the involvement of B-50 in neuropeptidergic CCK-8 release from large dense-cored vesicles. Indeed, in hippocampal presynaptic nerve terminals B-50 is predominantly present outside the active synaptic zone (Van Lookeren Campagne et al., 1990, 1992), where neuropeptide-containing large dense-cored vesicles are presumed to be released (Zhu et al., 1986; Verhage et al., 1991*b*).

In this article we describe the characterization of the Ca^{2+}-induced CCK-8 release from streptolysin O (SL-O)-permeated synaptosomes with respect to its Ca^{2+} and ATP dependency. In this permeated synaptosome system we subsequently tested the effects of two monoclonal antibodies directed against an N- and C-terminal epitope of B-50 and of PKC inhibitors on Ca^{2+}-induced CCK-8 release.

MATERIALS AND METHODS

Materials

Male Wistar rats weighing 100–120 g were used. Percoll and protein G were obtained from Pharmacia (Uppsala, Sweden); SL-O from Wellcome (Weesp, The Netherlands); bacitracin [a peptide mixture acting as a nonspecific, competitive protease inhibitor (McKelvy et al., 1976)], creatine phosphate, and creatine phosphokinase from Sigma (St. Louis, MO, U.S.A.); nonsulfated CCK-8 from Organon (Oss, The Netherlands); and sulfated CCK-8 from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were from Merck (Darmstadt, F.R.G.). The Ca^{2+}/EGTA buffers were calculated and prepared as described by Föhr et al. (1993).

IgG preparation

Ascites from pristane-primed BALB/c mice, injected with hybridomas NM2 and NM6, producing monoclonal anti-B-50 IgGs, were kindly provided by Dr. M. Mercken (Mercken et al., 1992). Anti-B-50 IgGs were purified by affinity chromatography on protein G, extensively dialyzed against 1,000 volumes of buffer A (124 m*M* NaCl and 5 m*M* PIPES, pH 6.8), and stored at −80°C. Titers of purified anti-B-50 IgG fractions were determined by a solid-phase ELISA using B-50 in lysed synaptosomes or purified dephosphorylated B-50 prepared as described by Andreasen et al. (1983). Total rabbit IgGs (ICN Biomedicals, Zoetermeer, The Netherlands) were used as control IgGs.

CCK-8 release from synaptosomes

Synaptosomes were prepared from rat cerebral cortex and isolated from the 15–23% interface (fraction 4) after density centrifugation on a Percoll–sucrose gradient (Dunkley et al., 1988). The synaptosomal preparation was washed three times in Krebs–Ringer buffer [124 m*M* NaCl, 5 m*M* KCl, 1.3 m*M* $MgSO_4$, 1.2 m*M* NaH_2PO_4, 2 m*M* $CaCl_2$, 10 m*M* D(+)-glucose, and 26 m*M* $NaHCO_3$, saturated with CO_2 and O_2 (5:95), pH 7.4] when intact synaptosomes were used or in buffer B [122.85 m*M* NaCl, 5 m*M* KCl, 2 m*M* $MgCl_2$, 1.15 m*M* NaH_2PO_4, 20 m*M* PIPES, and 5.6 m*M* D-(+)-glucose, oxygenated, pH 6.8] when synaptosomes were permeated. Synaptosomes were resuspended at 2 mg of protein/ml unless stated otherwise.

Intact synaptosomes (40 μg of protein in a final volume of 120 μl) were incubated in the presence of 1 mg/ml of bovine serum albumin and 0.2 mg/ml of protease inhibitor bacitracin for 5 min at 37°C. Krebs–Ringer buffer containing 20 m*M* K^+ was obtained by isotonic replacement of Na^+ by K^+. Krebs–Ringer buffer without Ca^{2+} was obtained by replacing 2 m*M* Ca^{2+} by 0.5 m*M* EGTA (final concentration). Incubations were stopped by centrifugation for 25 s at 10,000 *g*, and 100 μl of supernatant was added to 90% methanol and stored at −80°C for CCK-8 analysis.

In permeation experiments synaptosomes (40 μg of protein in a final volume of 120 μl) were incubated with 0.3 IU/ml of SL-O in buffer B supplemented with 2.0 m*M* ATP, 1 mg/ml of bovine serum albumin, 0.2 mg/ml of bacitracin, and the indicated concentration of free Ca^{2+} (buffered with 10 m*M* EGTA) for 5 min at 37°C, unless stated otherwise. Only in permeation experiments testing anti-B-50 IgGs were synaptosomes used at 20 μg of protein in 120 μl (final volume). Incubations were stopped by centrifugation for 25 s at 10,000 *g*, and 100 μl of supernatant was added to 90% methanol and stored at −80°C for CCK-8 analysis.

CCK-8 release was expressed in absolute amounts (femtomoles of CCK-8 per milligram of synaptosomal protein) and/or as a percentage of initial CCK-8 content. Initial CCK-8 content of synaptosomes was determined in the supernatant of synaptosomes lysed in the presence of 0.05%

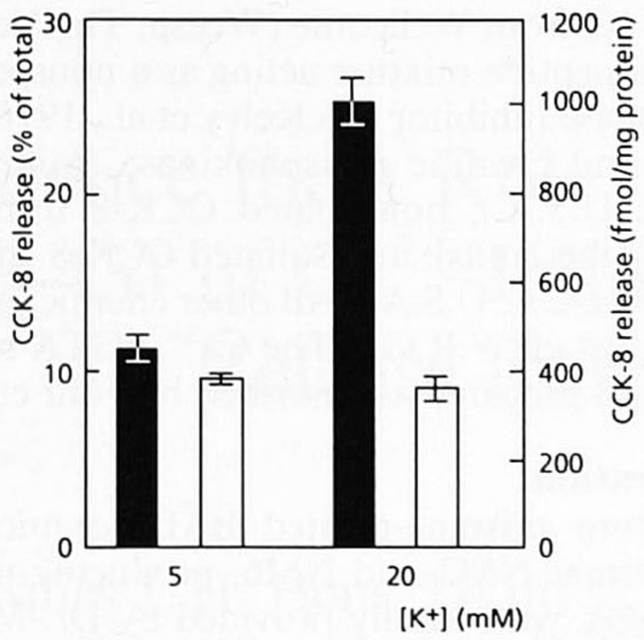

FIG. 1. Ca^{2+}-dependent CCK-8 release from K^+-depolarized intact synaptosomes. Intact synaptosomes were incubated either in the absence (□; 0.5 m*M* EGTA) or presence (■; 2 m*M*) of extracellular Ca^{2+} and stimulated with the indicated concentration of K^+ for 5 min at 37°C. CCK-8 release is expressed in absolute amounts as well as in a percentage of initial CCK-8 content. Data are mean ± SEM (bars) values (n = 8).

(vol/vol) Triton X-100 and 0.2 mg/ml of bacitracin at 37°C. Supernatant of lysed synaptosomes was obtained by centrifugation for 25 s at 10,000 *g*, added to 90% methanol, and stored at −80°C for CCK-8 analysis. Ca^{2+}-induced CCK-8 release was calculated by subtracting the percentage of CCK-8 release in the presence of 10^{-8} *M* Ca^{2+} from the percentage of CCK-8 release in the presence of 10^{-5} *M* Ca^{2+}.

CCK-8 analysis

CCK-8 release from synaptosomes incubated in the presence of bacitracin could be quantified by radioimmunoassay as described before (Verhage et al., 1991*a*), using antiserum L48 (Dockray, 1980). In short, CCK-8 was extracted and pelleted in ice-cold 90% methanol/10% water. Freeze-dried pellets were reconstituted in buffer C (20 m*M* sodium barbitone, 20 m*M* sodium azide, and 2.5 mg/ml of bovine serum albumin, pH 8.2) and incubated overnight with antiserum (diluted 1:6,000–8,000) at 4°C. Nonsulfated CCK-8 was used as the standard, and iodinated CCK-8 was used as the tracer. Cross-reactivity with sulfated CCK-8 was 100%. The antiserum has limited cross-reactivity with CCK-33 and high cross-reactivity with gastrins (Dockray, 1980). However, CCK-33 is not extracted in 90% methanol/10% water (Marley and Rehfeld, 1984), and in rat brain gastrins have only been found in small amounts in hypothalamoneurohypophyseal neurons (Marley et al., 1984).

Other determinations

Protein was quantified according to the technique of Bradford (1976) using bovine serum albumin as the standard. The amount of IgG was determined with total rabbit IgG as the reference. Data are mean ± SEM values, calculated on the basis of independent observations. Statistical analysis was performed with a two-tailed Student's *t* test, and $p < 0.05$ was regarded as significant.

RESULTS

Synaptosomes from rat cerebral cortex were highly purified by Percoll–sucrose gradient centrifugation and contained 6,000 ± 550 fmol of CCK-8/mg of synaptosomal protein (n = 30), when lysed in the presence of 0.2 mg/ml of the protease inhibitor bacitracin. Lysis in the absence of bacitracin resulted in a reduction of the measured CCK-8 content by about one-third. In control experiments we established that the CCK-8 content of lysed synaptosomes was independent of the free Ca^{2+} concentration present in the incubation medium.

On depolarization with 20 m*M* K^+, intact synaptosomes released 600 fmol of CCK-8/mg of synaptosomal protein within 5 min at 37°C (Fig. 1). This K^+-induced CCK-8 release was completely dependent on the presence of extracellular Ca^{2+}. Basal CCK-8 release in the presence of 5 m*M* K^+ was not affected by the extracellular Ca^{2+} concentration.

Subsequently, we investigated whether Ca^{2+}-induced CCK-8 release could be observed from SL-O-permeated synaptosomes. Therefore, we measured CCK-8 release at 10^{-8} and 10^{-5} *M* Ca^{2+} in the presence of increasing SL-O concentrations (Fig. 2). In the presence of 10^{-8} *M* Ca^{2+} the CCK-8 efflux (expressed as a percentage of total CCK-8 content) increased slightly, from 5% in the absence of SL-O to 7% in the presence of 0.5 IU/ml of SL-O (Fig. 2). In the presence of 10^{-5} *M* Ca^{2+} CCK-8 release was significantly higher than at 10^{-8} *M* Ca^{2+}, when permeated with SL-O concentrations of >0.1 IU/ml (Fig. 2). This additional CCK-8 release, referred to as Ca^{2+}-induced CCK-8 release, was maximal (~6%) at 0.4 IU/ml of SL-O. To minimize damage to intrasynaptosomal membranes, such as vesicle membranes, we used 0.3 IU/ml of SL-O in all subsequent experiments. Under identical permeation conditions Ca^{2+}-induced NA release was found to be 12% of total content (Hens et al., 1993).

The ATP dependency of Ca^{2+}-induced CCK-8 release is shown in Fig. 3. Ca^{2+}-induced CCK-8 release

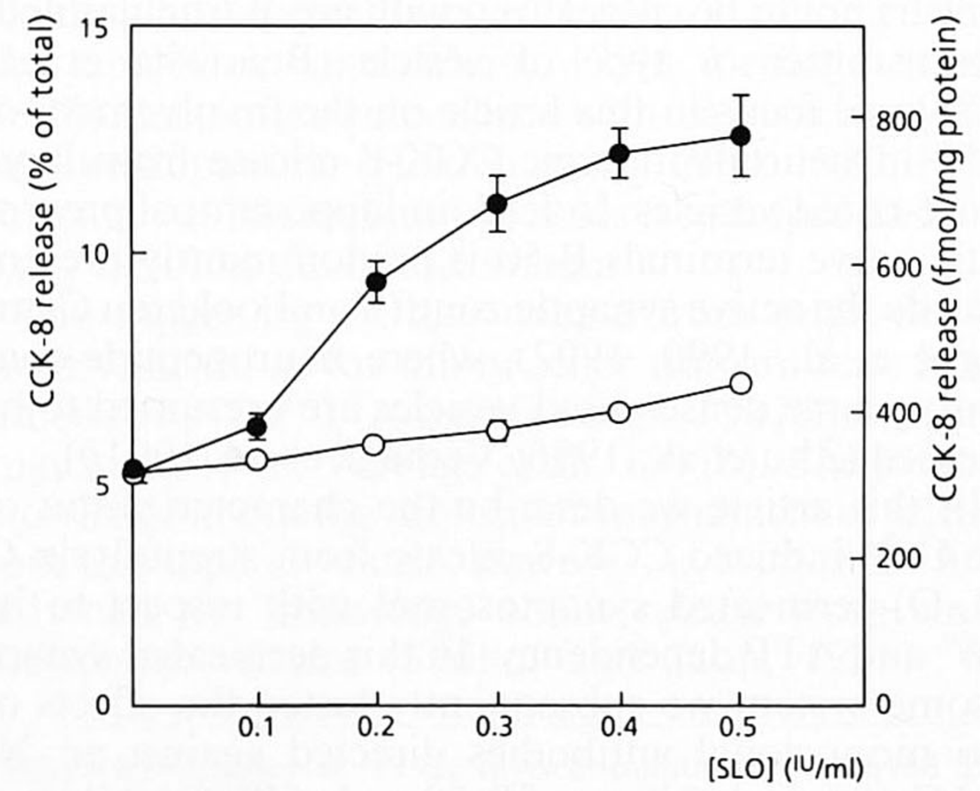

FIG. 2. Concentration-dependent permeation of synaptosomes by SL-O. Synaptosomes were permeated with indicated concentrations of SL-O in the presence of either 10^{-8} *M* (○) or 10^{-5} *M* (●) Ca^{2+}. CCK-8 release is expressed in absolute amounts as well as in a percentage of initial CCK-8 content. Data are mean ± SEM (bars) values from five independent experiments, each carried out in triplicate.

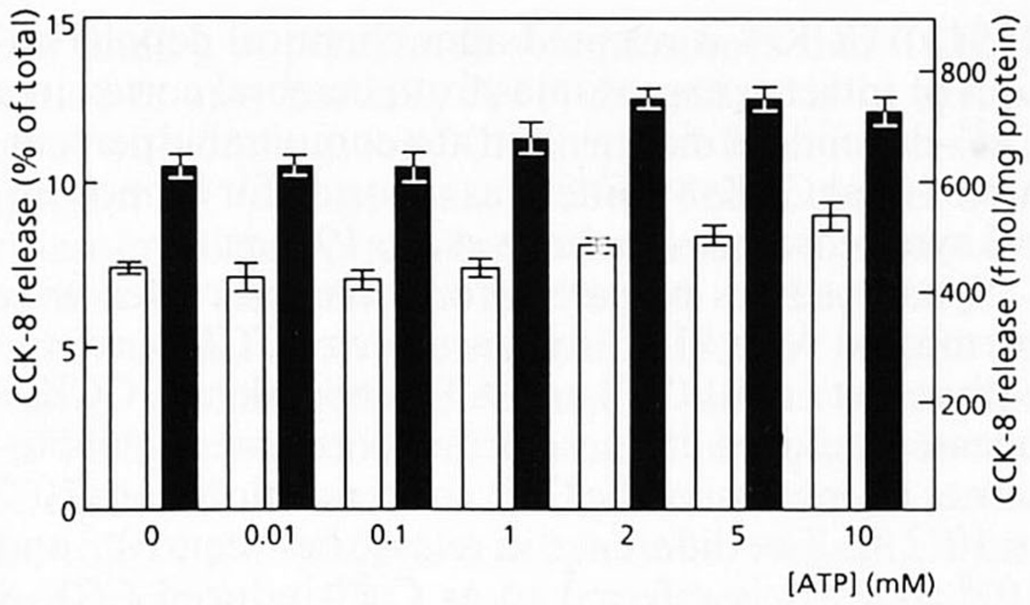

FIG. 3. ATP dependency of Ca^{2+}-induced CCK-8 release from SL-O-permeated synaptosomes. Synaptosomes were permeated with 0.3 IU/ml of SL-O at either 10^{-8} *M* (□) or 10^{-5} *M* (■) Ca^{2+} in the presence of 0–10 m*M* ATP. CCK-8 release is expressed in absolute amounts as well as in a percentage of initial CCK-8 content. Data are mean ± SEM (bars) values from five independent experiments, each carried out in triplicate. CCK-8 release at 10^{-8} and 10^{-5} *M* Ca^{2+} and the amount of Ca^{2+}-induced CCK-8 are significantly different between 0 and 2 m*M* ATP ($p < 0.05$, 0.01, and 0.05, respectively).

was only partially ATP dependent: In the absence of ATP, Ca^{2+}-induced release was 3% of the initial CCK-8 content (~70% of maximal Ca^{2+}-induced CCK-8 release). Addition of ATP resulted in a slight but significant increase of CCK-8 release irrespective of the Ca^{2+} concentration ($p < 0.05$). Ca^{2+}-induced release was maximal at 2.0 m*M* ATP (significantly different from 0 m*M* ATP; $p < 0.05$). Addition of an ATP-generating system [5 m*M* creatine phosphate and 5 units/ml of creatine phosphokinase (Helms et al., 1990)] in the presence of 2.0 m*M* ATP did not significantly affect Ca^{2+}-induced CCK-8 release. Further experiments were performed at 2.0 m*M* ATP.

Figure 4 shows the Ca^{2+} dependency of the CCK-8

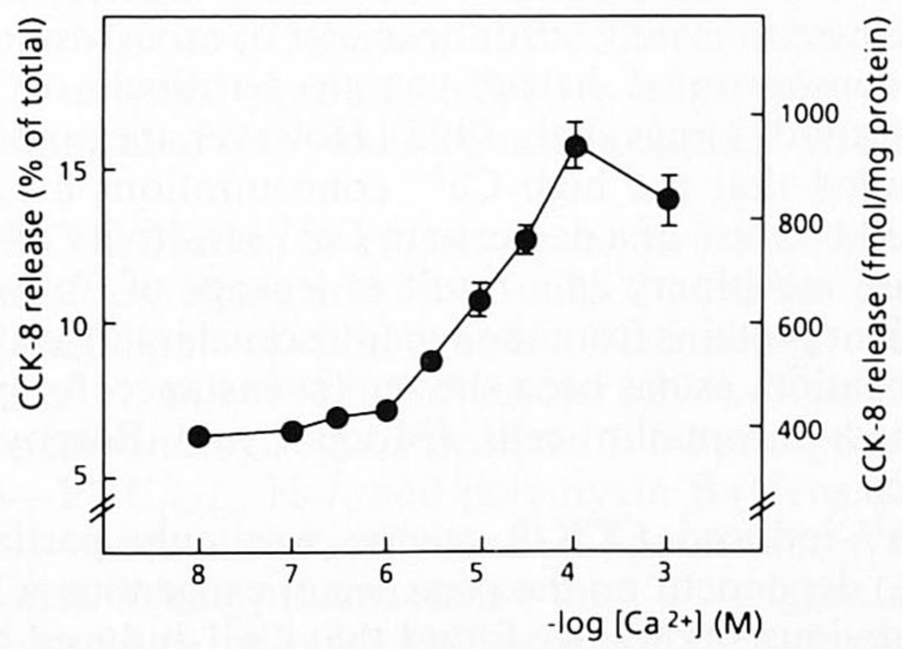

FIG. 4. Ca^{2+} dependency of CCK-8 release from SL-O-permeated synaptosomes. Synaptosomes were permeated with 0.3 IU/ml of SL-O in the presence of 2.0 m*M* ATP and the indicated free Ca^{2+} concentration. CCK-8 release is expressed in absolute amounts as well as in a percentage of initial CCK-8 content. Data are mean ± SEM (bars) values from seven independent experiments, each carried out in triplicate. Significant Ca^{2+}-induced CCK-8 release is observed at 3×10^{-6} *M* Ca^{2+} ($p < 0.01$).

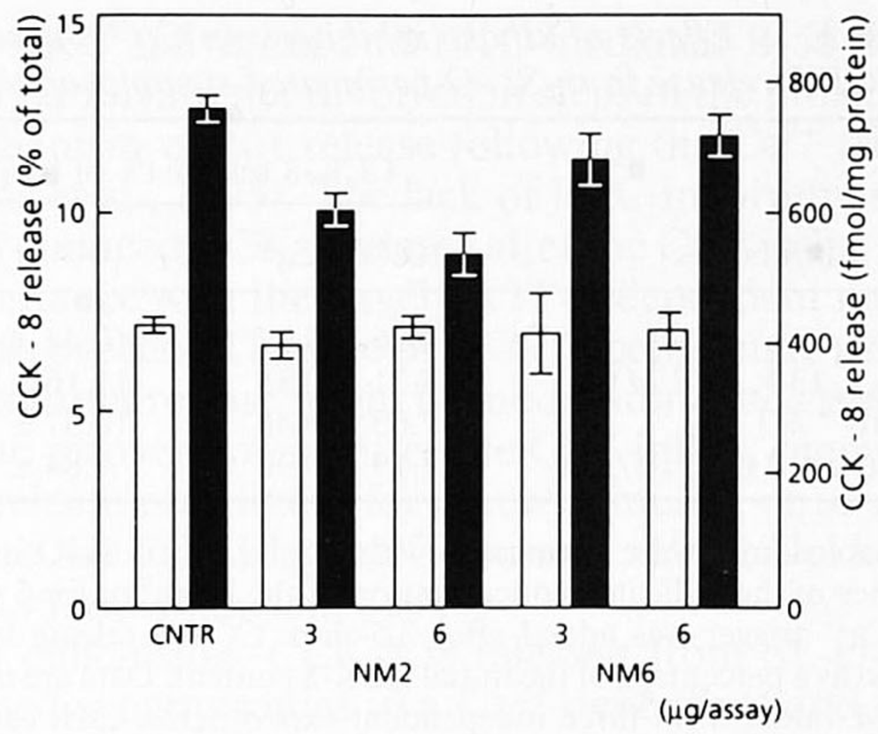

FIG. 5. Effects of N- (NM2) and C-terminally (NM6) directed monoclonal anti-B-50 IgGs on CCK-8 release from SL-O-permeated synaptosomes. Synaptosomes were permeated with 0.3 IU/ml of SL-O in the presence of 10^{-8} (□) or 10^{-5} *M* (■) Ca^{2+}. Anti-B-50 IgGs were present at the indicated amount (in a final volume of 120 µl) throughout the experiment. CCK-8 release is expressed in absolute amounts as well as in a percentage of initial CCK-8 content. Data are mean ± SEM (bars) values from three independent experiments, each carried out in triplicate. Significant inhibition of CCK-8 release at 10^{-5} *M* Ca^{2+} is observed at 3 µg of IgG NM2 ($p < 0.05$). CNTR, control.

release from SL-O-permeated synaptosomes. Significant stimulation of CCK-8 release was first seen at 3×10^{-6} *M* Ca^{2+} ($p < 0.01$) compared with 10^{-8} *M* Ca^{2+} [basal intrasynaptosomal Ca^{2+} concentration (Verhage et al., 1988)]. Half-maximal stimulation was found at 10^{-5} *M* Ca^{2+}. Maximal stimulation was observed at 10^{-4} *M* Ca^{2+}, being 9.5% (~600 fmol of CCK/mg of synaptosomal protein) of the total synaptosomal CCK-8 content.

Two different monoclonal anti-B-50 IgGs, NM2 and NM6, which recognize both phospho- and dephospho-B-50 (Mercken et al., 1992), were tested for their effect on Ca^{2+}-induced CCK-8 release. We have shown previously that NM2 inhibits B-50 phosphorylation in synaptosomal plasma membranes and permeated synaptosomes, whereas NM6 is without effect (J. J. H. Hens et al., submitted for publication). Addition of the C-terminally directed anti-B-50 IgG NM6 (6 µg in a final volume of 120 µl) did not affect Ca^{2+}-induced CCK-8 release (Fig. 5). Addition of N-terminally directed anti-B-50 IgG NM2, however, inhibited Ca^{2+}-induced CCK-8 in a concentration-dependent manner, half maximally at 3 µg of IgG ($p < 0.05$) and almost completely at 6 µg of IgG (Fig. 5). Basal CCK-8 release in the presence of 10^{-8} *M* Ca^{2+} was not affected either by NM2 or by NM6. When tested in the solid-phase ELISA, NM6 had at least a threefold higher titer for B-50 in lysed synaptosomes than NM2 and at least a 10-fold higher titer for purified dephosphorylated B-50. A similar difference in titer between NM2 and NM6 was reported for B-50 immunostaining of western blots (Mercken et al., 1992). Therefore,

TABLE 1. *Effects of kinase inhibitors on Ca^{2+}-induced CCK-8 release from SL-O-permeated synaptosomes*

Inhibitor	CCK-8 release (% of total)	
	10^{-8} *M* Ca^{2+}	10^{-5} *M* Ca^{2+}
Control	8.26 ± 0.27	10.44 ± 0.32
PKC_{19-36} (3×10^{-5} *M*)	8.31 ± 0.36	11.16 ± 0.50
H-7 (10^{-4} *M*)	7.65 ± 0.46	10.37 ± 0.40
Polymyxin B (200 IU/ml)	7.19 ± 0.36	7.84 ± 0.42

Synaptosomes were permeated with 0.3 IU/ml of SL-O in the presence of the indicated concentration of the inhibitor for 5 min. The Ca^{2+} trigger was added after 2.5 min. CCK-8 release is expressed as a percentage of the initial CCK-8 content. Data are mean ± SEM values from three independent experiments, each carried out in triplicate.

the lack of effect of NM6 cannot be attributed to a lower affinity toward B-50. Control rabbit total IgGs did not affect Ca^{2+}-induced CCK-8 release. The degree of permeation of synaptosomes, determined by the efflux of cytosolic marker protein lactate dehydrogenase, was not affected by any of the anti-B-50 IgGs.

To investigate the involvement of PKC and PKC-mediated B-50 phosphorylation [which is inhibited by the anti-B-50 IgG NM2 (De Graan et al., 1992; J. J. H. Hens et al., submitted for publication)] in Ca^{2+}-induced CCK-8 release from SL-O-permeated synaptosomes, we tested the effect of three kinase inhibitors at a concentration known to inhibit B-50 phosphorylation in SL-O-permeated synaptosomes (Hens et al., 1993): PKC_{19-36} at 3×10^{-5} *M*, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) at 10^{-4} *M*, and polymyxin B at 200 IU/ml (Table 1). When added simultaneously with the Ca^{2+} trigger, Ca^{2+}-induced CCK-8 release was not affected either by PKC_{19-36} or by H-7, whereas polymyxin B treatment resulted in complete inhibition (data not shown). Preincubation for 2.5 min with PKC inhibitors PKC_{19-36} and H-7 before application of the Ca^{2+} trigger did not affect Ca^{2+}-induced CCK-8 release from SL-O-permeated synaptosomes either, whereas release was inhibited completely after preincubation with polymyxin B (Table 1).

DISCUSSION

To study the role of B-50 in the molecular mechanism underlying vesicular neuropeptide release, we developed a release assay for CCK-8 from permeated synaptosomes. CCK-8 was selected as a representive neuropeptide for its abundant presence and widespread occurrence in rat cerebral cortex (Rehfeld, 1985). In accordance with its distribution in the CNS (Marley et al., 1984), CCK-8 levels in synaptosomes from rat cerebral cortex (6,000 ± 550 fmol/mg of protein; n = 30) are 2.5-fold higher than those reported for hippocampal synaptosomes (Verhage et al., 1991*a*). CCK-8 is released after chemical depolarization of intact synaptosomes from cerebral cortex in a Ca^{2+}-dependent manner and at a comparable percentage of total CCK-8 content as reported for hippocampal synaptosomes (Verhage et al., 1991*a*).

Synaptosomes prepared from cerebral cortex were permeated with SL-O to characterize CCK-8 release with respect to its Ca^{2+} and ATP dependency. CCK-8 release could be induced from permeated synaptosomes by increasing the Ca^{2+} concentration from 10^{-8} to 10^{-5} *M*. The difference in release between 10^{-5} and 10^{-8} *M* Ca^{2+} is referred to as Ca^{2+}-induced CCK-8 release. Ca^{2+}-induced CCK-8 release was maximal at SL-O concentrations of >0.3 IU/ml, as was observed for Ca^{2+}-induced NA release from SL-O-permeated synaptosomes in previous studies (De Graan et al., 1991). As a result of SL-O permeation of synaptosomes, basal NA release (in the presence of 10^{-8} *M* Ca^{2+}) increased (De Graan et al., 1991), whereas basal CCK-8 release was hardly affected. This confirms the vesicular nature of Ca^{2+}-induced CCK-8 release from SL-O-permeated synaptosomes and indicates that CCK-8-containing large dense-cored vesicles are much less sensitive to SL-O treatment than NA-containing vesicles. Therefore, we consider the SL-O-permeated synaptosome as a valid model system to study the mechanism of Ca^{2+}-induced CCK-8 release.

CCK-8 release from SL-O-permeated synaptosomes can be induced by elevation of the free Ca^{2+} concentration. Similarly, Ca^{2+}-dependent vasopressin release from permeated rat neurohypophysis nerve endings was reported (Cazalis et al., 1987). Our data suggest that CCK-8 release is initiated at a slightly higher Ca^{2+} concentration (3×10^{-6} *M*) than observed for NA [10^{-6} *M* (Hens et al., 1993)], but we cannot rule out that this is due to a difference in the sensitivity of the detection assays for the transmitters. Maximal Ca^{2+}-induced release for CCK-8 as well as for NA was observed at ~10^{-4} *M* Ca^{2+}. These Ca^{2+} concentrations may occur near sites of exocytosis and are demonstrated in presynaptic terminals of the giant squid (Llinás et al., 1992). However, it cannot be excluded that the high Ca^{2+} concentrations are required because of a decrease in Ca^{2+} sensitivity of the release machinery as a result of leakage of Ca^{2+}-dependent proteins from the synaptosomal interior after permeation, as has been shown, for instance, for permeated chromaffin cells (Morgan and Burgoyne, 1992).

Ca^{2+}-induced CCK-8 release was only partially (30%) dependent on the presence of exogenous ATP. In previous studies, we found that Ca^{2+}-induced NA release from permeated synaptosomes is largely (>60%) ATP dependent (Dekker et al., 1991*b*; Hens et al., 1993). These observations are in accordance with the fact that peptide-containing vesicles are formed in the cell body and are not, like catecholamine-containing vesicles, dependent on ATPase-mediated vesicle storage in the nerve terminal itself.

Our finding in permeated synaptosomes that CCK-8 release is only partially ATP dependent is in agreement with findings for CCK-8 release from intact synaptosomes (Verhage et al., 1991*a*). This indicates that kinase-mediated phosphorylation events are not important in steps in the mechanism of large dense-cored vesicle fusion occurring after the Ca^{2+} influx. Of course, neither the involvement of kinases before the Ca^{2+} trigger nor the involvement of phosphatases can be excluded. To obtain more specific information on the role of phosphorylation reactions, we studied the involvement of PKC and its presynaptic substrate B-50 in Ca^{2+}-induced CCK-8 release, using anti-B-50 IgGs. We have shown previously that affinity-purified anti-B-50 IgGs, introduced into SL-O-permeated synaptosomes, inhibit PKC-mediated B-50 phosphorylation and Ca^{2+}-induced NA release (Dekker et al., 1989, 1991*b*; Hens et al., 1993).

Here we show that the monoclonal anti-B-50 IgG NM2 inhibits Ca^{2+}-induced CCK-8 release in a concentration-dependent manner. The anti-B-50 IgG NM2 is directed against an epitope of B-50 located in the proposed calmodulin-binding domain of B-50, extending from amino acid 39 to 51, because its ability to inhibit PKC-mediated B-50 phosphorylation is lost after preincubation with corresponding synthetic B-50 peptides (J. J. H. Hens and P. N. E. De Graan, unpublished data). The monoclonal anti-B-50 IgG NM6 and total rabbit IgGs, used as negative controls, are without effect on Ca^{2+}-induced CCK-8 release. The monoclonal IgG NM6 is directed against an epitope located between residue 132 and 214 of B-50 and does not inhibit B-50 phosphorylation (De Graan et al., 1992; J. J. H. Hens et al., submitted for publication). The inability of NM6 to inhibit Ca^{2+}-induced CCK-8 release shows that nonspecific steric hindrance by the antibody is not likely to cause the inhibition of Ca^{2+}-induced CCK-8 release observed with the anti-B-50 IgG NM2. Although we cannot exclude a function of the C-terminus of B-50, we conclude that the N-terminal portion of B-50 is important in the mechanism of Ca^{2+}-induced neuropeptide CCK-8 release.

PKC activation is closely associated with neurotransmitter release and is considered to be an important regulator of this process (Kaczmarek, 1987; Dekker et al., 1991*a*; Robinson, 1991). Therefore, three inhibitors of PKC-mediated B-50 phosphorylation—PKC_{19-36}, H-7, and polymyxin B (Hens et al., 1993)—were introduced into SL-O-permeated synaptosomes. When present before the Ca^{2+} trigger, only polymyxin B inhibited Ca^{2+}-induced CCK-8 release completely, whereas PKC_{19-36} and H-7 were without effect. Polymyxin B was used as a positive control for the inhibition of Ca^{2+}-induced CCK-8 release, because it was reported to inhibit calmodulin-dependent processes more potently than PKC-mediated processes (Hegemann et al., 1991). These observations are in line with earlier findings, demonstrating that PKC activation and PKC-mediated B-50 phosphorylation are not involved in steps in the molecular mechanism of NA release following the Ca^{2+} trigger (Hens et al., 1993). The lack of PKC involvement in Ca^{2+}-induced CCK-8 release after the Ca^{2+} influx is in accordance with the largely ATP-independent nature of this process. The role of PKC in peptidergic neurotransmitter release might be modulatory and essential in the recovery phase after the Ca^{2+} influx, preparing the release machinery for a new stimulus, or in steps before the Ca^{2+} trigger (Kaczmarek, 1987; Dekker et al., 1991*a*; Robinson, 1991).

In conclusion, CCK-8 is released from SL-O-permeated synaptosomes in a Ca^{2+}-dependent and only partially ATP-dependent manner. We established a causal relationship between B-50 and the Ca^{2+}-induced CCK-8 release from large dense-cored vesicles. Activation of PKC is not likely to be part of the molecular events that occur after the Ca^{2+} influx and result in fusion of CCK-8-containing vesicles with the presynaptic membrane. In future experiments it will be of interest to test whether B-50 is involved in Ca^{2+}-induced release from small clear-cored vesicles as well, because it has been reported that the degree of B-50 phosphorylation is elevated after high-frequency stimulation of hippocampus slices resulting in long-term potentiation (Gianotti et al., 1992), a process in which glutamate plays an important role.

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