Journal of Neurochemistry
Raven Press, Ltd., New York

Noradrenaline Release from Streptolysin O-Permeated Rat Cortical Synaptosomes: Effects of Calcium, Phorbol Esters, Protein Kinase Inhibitors, and Antibodies to the Neuron-Specific Protein Kinase C Substrate B-50 (GAP-43)

Lodewijk V. Dekker, Pierre N. E. De Graan, Pim Pijnappel, A. Beate Oestreicher, and Willem Hendrik Gispen

Division of Molecular Neurobiology, Rudolf Magnus Institute and Institute of Molecular Biology and Medical Biotechnology, Utrecht, The Netherlands

Abstract: We studied the molecular mechanism of noradrenaline release from the presynaptic terminal and the involvement of the protein kinase C substrate B-50 (GAP-43) in this process. To gain access to the interior of the presynaptic terminal, we searched for conditions to permeate rat brain synaptosomes by the bacterial toxin streptolysin O. A crude synaptosomal/mitochondrial preparation was preloaded with [^{3}H]noradrenaline. After permeation with 0.8 IU/ml streptolysin O, noradrenaline efflux could be induced in a concentration-dependent manner by elevating the free Ca^{2+} concentration from 10^{-8} to 10^{-5} *M*. Efflux of the cytosolic marker protein lactate dehydrogenase was not affected by this increase in Ca^{2+}. Ca^{2+}-induced efflux of noradrenaline was largely dependent on the presence of exogenous ATP. Changing the Na^+/K^+ ratio in the buffer did not affect Ca^{2+}-induced noradrenaline release. Release of noradrenaline could also be evoked by phorbol esters, indicating the involvement of protein kinase C. Ca^{2+}- and phorbol ester-induced release were not additive at higher phorbol ester concentrations ($>10^{-7}$ *M*). We compared the sensitivities of Ca^{2+}- and phorbol ester-induced release of noradrenaline to the protein kinase inhibitors H-7 and polymyxin B and to antibodies raised against synaptic protein kinase C substrate B-50. Ca^{2+}-induced release was inhibited by B-50 antibodies and polymyxin B, but not by H-7; phorbol ester-induced release was inhibited by polymyxin B and by H-7, but only marginally by antibodies to B-50. We suggest that phorbol esters and Ca^{2+} stimulate noradrenaline release through different mechanisms and that the essential role of B-50 in Ca^{2+}-induced noradrenaline release may involve other properties of B-50 besides protein kinase C–mediated phosphorylation. **Key Words:** Noradrenaline release—Streptolysin O—Synaptosome—Protein kinase C—B-50—GAP-43. **Dekker L. V. et al.** Noradrenaline release from streptolysin O-permeated rat cortical synaptosomes: Effects of calcium, phorbol esters, protein kinase inhibitors, and antibodies to the neuron-specific protein kinase C substrate B-50 (GAP-43). *J. Neurochem.* **56,** 1146–1153 (1991).

It is widely accepted that release of most neurotransmitters from the presynaptic nerve terminal requires fusion of transmitter-containing vesicles with the presynaptic membrane and that Ca^{2+} is a trigger for this event. However, little is known about the molecular mechanisms. A major difficulty in resolving these mechanisms has been the inaccessibility of the interior of the presynaptic terminal to experimental manipulation. Therefore, a method is required that renders the presynaptic terminal permeable for ions and macromolecules, without affecting the exocytotic machinery. Several permeation techniques, including erythrocyte ghost fusion (Schweizer et al., 1989), electropermeation (Knight and Scrutton, 1986), detergents (Peppers and Holz, 1986), freeze-thawing (Nichols et al., 1989), and toxins (Ahnert-Hilger et al., 1985*a*; Howell and Gomperts, 1987), have been developed to study stimulus-secretion coupling in secretory cells such as mast cells (Howell and Gomperts, 1987; Howell et al., 1987, 1989), chromaffin cells (Kenigsberg and Tri-

Received April 27, 1990; revised manuscript received July 18, 1990; accepted September 25, 1990.

Address correspondence and reprint requests to Dr. P. N. E. De Graan at Division of Molecular Neurobiology, Rudolf Magnus Institute, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Abbreviations used: Ig, immunoglobulin; LDH, lactate dehydrogenase; NA, noradrenaline; PAGE, polyacrylamide gel electrophoresis; PDB, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; SDS, sodium dodecyl sulfate; SL-O, streptolysin O; SPM, synaptosomal plasma membrane.

faro, 1985; Lee and Holz, 1986; Bittner and Holz, 1988), and PC12 cells (Ahnert-Hilger et al., 1985*a*; Peppers and Holz, 1986). In these permeated secretory cells, release could be induced by elevation of the Ca^{2+} concentration in the permeation buffer. Ca^{2+}-induced release was found to be dependent on Mg^{2+}, and ATP-dependent as well as ATP-independent release have been described (Reynolds et al., 1982; Ahnert-Hilger et al., 1985*a,b*, 1987, 1989; Peppers and Holz, 1986; Ahnert-Hilger and Gratzl, 1987).

Recently, we have permeated synaptosomes, isolated pinched-off presynaptic nerve terminals, with the toxin streptolysin O (SL-O) (Dekker et al., 1989*a*). As in mast, chromaffin, and PC12 cells, noradrenaline (NA) release from SL-O-permeated synaptosomes could be induced by elevation of external Ca^{2+} (Dekker et al., 1989*a*). We have used these SL-O-permeated synaptosomes to introduce antibodies to B-50, a nervous tissue-specific substrate of protein kinase C (PKC), associated with the cytosolic face of the presynaptic membrane (Zwiers et al., 1980; Sörensen et al., 1981; Kristjansson et al., 1982; Aloyo et al., 1983; Gispen et al., 1985; De Graan et al., 1988, 1989; Van Lookeren Campagne et al., 1989). The B-50 protein is identical to the growth-associated protein GAP-43, to the calmodulin-binding protein neuromodulin, and to protein F1, which is implicated in long-term potentiation (Basi et al., 1987; Cimler et al., 1987; Karns et al., 1987; Nielander et al., 1987; Rosenthal et al., 1987). In a series of studies we have shown that the degree of PKC-mediated phosphorylation of B-50 in hippocampal slices and synaptosomes is correlated with transmitter release (Dekker et al., 1989*b*, 1990*a,b*). Based on these correlative studies and the fact that phorbol esters that stimulate PKC enhance neurotransmitter release (Allgaier et al., 1986; Wakade et al., 1986; Versteeg and Florijn, 1987), we have suggested that PKC-mediated B-50 phosphorylation may be involved in the regulation of neurotransmitter release. Indeed, antibodies to B-50, which inhibit PKC-mediated B-50 phosphorylation, completely inhibited Ca^{2+}-induced release of NA from SL-O-permeated synaptosomes (Dekker et al., 1989*a*).

In the present study we characterized NA release from a SL-O-permeated cortical synaptosome preparation with respect to permeation conditions, Ca^{2+} sensitivity, and ATP dependency and investigated the involvement of PKC and its substrate B-50 in NA release using phorbol esters, protein kinase inhibitors, and antibodies to B-50. Ca^{2+} as well as phorbol esters induced NA release from permeated synaptosomes. The protein kinase inhibitors H-7 and polymyxin B and the B-50 antibodies differentially affected Ca^{2+}- and phorbol ester-induced NA release.

MATERIALS AND METHODS

Materials

Male Wistar rats (TNO, Zeist, the Netherlands) of 120 g were used throughout the experiments. SL-O was obtained from the Wellcome Trust (Weesp, the Netherlands). ATP was from Boehringer (Mannheim, F.R.G.). [γ-^{32}P]ATP (specific activity 3,000 Ci/mmol) and L-[7,8-^{3}H]NA (specific activity 34 Ci/mmol) were from Amersham (U.K.). Phorbol 12,13-dibutyrate (PDB), 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12-myristate 13-acetate (PMA), polymyxin B, and H-7 were from Sigma (St. Louis, MO, U.S.A.). Pansorbin was from Calbiochem (La Jolla, CA, U.S.A.). B-50 antibodies were prepared as described in Oestreicher et al. (1983). Total rabbit immunoglobulin (Ig) G from Miles (U.K.) was used as control IgG. The Ca^{2+}/EGTA buffers used to obtain accurate Ca^{2+} concentrations in the micromolar range were calculated and prepared as described by Howell and Gomperts (1987). Synaptosomal plasma membranes (SPMs) were prepared as described previously (Kristjansson et al., 1982).

Release of [^{3}H]NA

A crude synaptosomal/mitochondrial preparation, further referred to as synaptosomes, was prepared from rat cortex as described by De Langen et al. (1979). Cerebral cortex was homogenized (10% wt/vol) in 0.32 *M* sucrose. The homogenate was centrifuged for 10 min at 1,000 *g*. Two-milliliter S_1 fractions were diluted with 3 ml buffer A (123 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 1.15 m*M* NaH_2PO_4, 5.6 m*M* glucose, 20 m*M* PIPES, pH 6.8, gassed with O_2/CO_2, 95%/5%) and incubated for 15 min at 37°C. Then 2.5 μCi NA was added, incubation was continued for 15 min, and the synaptosomes were centrifuged for 20 min at 600 *g*. The resulting pellet (P_2) was washed twice in buffer A without $CaCl_2$ and resuspended (1 mg protein/ml).

For measurement of NA release, prelabeled synaptosomes (washed P_2) were incubated at 37°C in buffer A containing Na_2ATP and EGTA-buffered Ca^{2+} in concentrations as indicated (final volume 120 μl). The release reaction was started by addition of synaptosomes (20 μg protein) to the reaction mixture (containing SL-O and compounds to be tested) and terminated after 5 min by centrifugation for 30 s at 10,000 *g*. The supernatant (90 μl) was collected and counted in a Packard model 2,000 CA liquid scintillation counter with Picofluor as scintillation cocktail. For measurement of lactate dehydrogenase (LDH) efflux, synaptosomes were treated as for NA release except that the amount of LDH was determined in the supernatants according to Bergmeyer and Bernt (1970). NA release was expressed as percentage of total [^{3}H]NA incorporated. Total NA incorporation was determined by measuring ^{3}H incorporation in 20 μg prelabeled washed synaptosomes.

Protein phosphorylation

Two-milliliter S_1 fractions were diluted with 3 ml buffer A containing 10^{-5} *M* EGTA–buffered Ca^{2+} (instead of 2 m*M* Ca^{2+}) and centrifuged for 20 min at 600 *g*. The resulting pellet (P_2) was resuspended in buffer A (0.5 mg protein/ml). In studies using lysed synaptosomes, the P_2 pellet was lysed in double-distilled H_2O and the lysate was adjusted to buffer A (0.5 μg protein/μl).

For the phosphorylation experiments 10 μg synaptosomes was preincubated at 37°C in buffer A with SL-O at the indicated concentration in the presence or absence of the compounds to be tested. After 10 min (unless otherwise indicated), phosphorylation was started by the addition of 7.5 μ*M* [γ-^{32}P]ATP. The reaction was terminated after 15 s by the addition of a denaturing stop solution [final concentration: 2% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol,

5% (vol/vol) 2-mercaptoethanol, 0.001% (wt/vol) bromophenol blue, 62.5 m*M* Tris-HCl, pH 6.8]. Immunoprecipitation of B-50 from phosphorylated synaptosomes was performed according to De Graan et al. (1989). Protein phosphorylation was analyzed by 11% SDS polyacrylamide gel electrophoresis (PAGE) and autoradiography followed by densitometric scanning (Kristjansson et al., 1982).

Other determinations

Proteins were determined according to Bradford (1976) with bovine serum albumin as a standard. For IgG determination, total rabbit IgG was used as a reference. Statistical analysis was performed with a two-tailed Student's *t* test.

RESULTS

Optimal permeation conditions

Synaptosomes were permeated using the bacterial toxin SL-O. To monitor the degree of permeation of the synaptosomes, phosphorylation of B-50 (an intrasynaptosomal substrate of PKC) was measured using exogenous [γ-^{32}P]ATP as phosphate donor. Figure 1A shows that SL-O treatment of synaptosomes enhanced the phosphorylation of a protein comigrating with a B-50 marker (Fig. 1A, lane S). The increase in B-50 phosphorylation after SL-O treatment was confirmed by immunoprecipitation (Fig. 1B). The effect of SL-O permeation on B-50 phosphorylation measured by immunoprecipitation was concentration dependent. Densitometric analysis of ^{32}P incorporation after immunoprecipitation revealed that the minimal effective dose of SL-O (0.4 IU/ml) increased B-50 phosphorylation by 240 ± 8% (n = 6, $p < 0.001$) as compared with untreated controls. At 1.0 IU/ml SL-O, B-50 phosphorylation was significantly higher than at 0.4 IU/ml (420 ± 11%; n = 6, $p < 0.001$). In the absence of SL-O, B-50 phosphorylation was so low that it could be detected only after immunoprecipitation, indicating that the preparation consisted predominantly of intact synaptosomes, inaccessible to [γ-^{32}P]ATP. The increase in B-50 phosphorylation after SL-O treatment could not be attributed to a direct effect of SL-O on PKC, as SL-O did not stimulate B-50 phosphorylation in synaptosomes that had been lysed with H_2O prior to the SL-O treatment (not shown). In Fig. 2 the time dependency of the SL-O effect on synaptosomal protein phosphorylation is shown. Maximal B-50 phosphorylation (251 ± 6% as compared with controls) was reached after a 5-min treatment with 0.4 IU/ml SL-O. Therefore, a 5-min SL-O treatment was used in further experiments. These data show that SL-O (at concentrations higher than 0.4 IU/ml) renders the synaptosomes permeable to [γ-^{32}P]ATP.

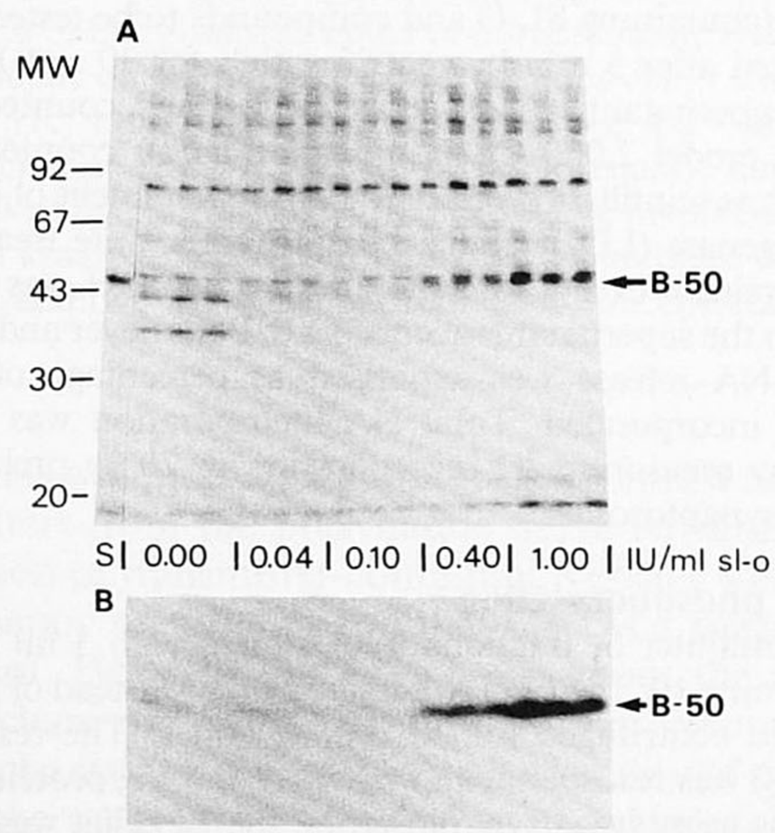

FIG. 1. Autoradiogram showing the effect of SL-O permeation on synaptosomal protein phosphorylation. Synaptosomes were incubated with 0.00–1.00 IU/ml SL-O for 5 min at 37°C. Phosphorylation was started by addition of 7.5 μM [γ-^{32}P]ATP and stopped after 15 s by addition of SDS-containing stop mix. **A:** Total protein phosphorylation, analyzed by SDS-PAGE and autoradiography. **B:** Synaptosomes phosphorylated as in A were subjected to B-50 immunoprecipitation prior to SDS-PAGE and autoradiography. Lane marked "S" shows the endogenous phosphorylation pattern of SPMs in which B-50 is the most prominent phosphoprotein. Samples were run in triplicate.

We have recently shown (Dekker et al., 1989*a*) that depolarization-induced NA release can be mimicked in SL-O-permeated synaptosomes by increasing the free Ca^{2+} concentration from 10^{-7} *M* (the normal basal intrasynaptosomal Ca^{2+} level; Verhage et al., 1988) to 10^{-5} *M* (a Ca^{2+} concentration allowing release from permeated secretory cells; see introductory section). Ca^{2+}-induced NA release was maximal (~3.8% of total NA incorporation) at SL-O concentrations between 0.8 and 1.0 IU/ml (Dekker et al., 1989*a*). From these studies we conclude that it is possible to permeate synaptosomes with SL-O, creating pores that allow access of impermeable molecules such as ATP and Ca^{2+} without affecting the release machinery. In subsequent experiments we used a 5-min treatment with 0.8 IU/ml SL-O to further characterize Ca^{2+}-induced NA release.

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

NA-prelabeled synaptosomes were permeated at different Ca^{2+} concentrations (Fig. 3). Net Ca^{2+}-dependent release of NA was calculated by subtracting NA efflux in the presence of 10^{-8} *M* Ca^{2+} from NA efflux in the presence of the indicated concentration of free Ca^{2+}. Ca^{2+} induced a significant increase in NA release at concentrations of $>3 \times 10^{-7}$ *M*. At 10^{-5} *M* Ca^{2+}, net Ca^{2+}-dependent NA release amounted to ~3% of the total NA incorporated. Although Ca^{2+}-induced release appears not to be maximal at 10^{-5} *M*, higher concentrations could not be tested, because above 10^{-5} *M* Ca^{2+}, the Ca^{2+}/EGTA buffer system is not reliable. The efflux of the cytosolic marker molecule LDH from the permeated synaptosomes was sixfold higher in the presence than in the absence of 0.8 IU/ml SL-O, indicating that SL-O indeed permeates the synaptosomes (not shown). Permeation in the presence of various concentrations of Ca^{2+} did not affect LDH efflux (Fig. 3).

Based on the work of others (Howell and Gomperts, 1987), we used a buffer system with standard extra-

cellular Na^+ and K^+ concentrations. We tested whether changing the concentrations of these cations toward more intracellular composition would affect the sensitivity of the NA release mechanism for Ca^{2+}. As shown in Table 1, Ca^{2+}-induced release of NA was not affected by changing the Na^+/K^+ ratio in the buffer. Apparently, Na^+ and K^+ can be mutually exchanged.

To investigate the ATP dependency of Ca^{2+}-induced NA release, we tested the effect of various concentrations of ATP (Fig. 4). In the absence of ATP, Ca^{2+}-induced NA release was 1.4%. Ca^{2+}-induced release was significantly enhanced at 0.1 m*M* ATP (2.6%) and was further increased to 3.9% at 5 m*M* ATP, the highest dose tested (Fig. 4). To control the effect of ATP on SL-O activity, efflux of LDH was measured. Both at 10^{-7} and at 10^{-5} *M* Ca^{2+}, ATP had no effect on the efflux of LDH from the SL-O-permeated synaptosomes (not shown).

Involvement of PKC and B-50 in Ca^{2+}- and phorbol ester-induced NA release

At 10^{-7} *M* Ca^{2+}, the phorbol ester PMA stimulated efflux of NA from permeated synaptosomes in a concentration-dependent manner (Fig. 5). The lowest dose inducing significant stimulation was 10^{-8} *M*. Similar results were obtained with PDB. The effect of phorbol esters was stereospecific as the phorbol ester 4α-PDD had no effect on NA efflux. At 10^{-5} *M* Ca^{2+}, PMA stimulated NA release at 10^{-7} and 10^{-6} *M*. At PMA concentrations up to 10^{-7} *M*, elevation of the Ca^{2+} concentration from 10^{-7} to 10^{-5} *M* significantly enhanced PMA-induced release. At 10^{-6} *M* PMA, no further stimulation of NA release was observed by elevating the Ca^{2+} concentration.

Another indication that PKC is involved in NA release from permeated synaptosomes is the fact that antibodies to the PKC substrate B-50, inhibiting the phosphorylation of B-50, completely inhibit Ca^{2+}-induced release of NA (Fig. 6). To our surprise, the B-50 antibodies only slightly inhibited 10^{-7} *M* PMA-induced NA release (14%; $p < 0.01$) from the same synaptosome preparation at 10^{-7} *M* Ca^{2+}. At 10^{-5} *M* Ca^{2+}, the antibodies selectively and completely inhibited only that part of the release that can be accounted for by the increase in Ca^{2+} concentration. Total rabbit IgG or heat-inactivated B-50 antibodies affected neither B-50 phosphorylation nor NA release in these experiments (Fig. 6).

Next we tested the effects of the protein kinase inhibitors H-7 (Hidaka et al., 1984) and polymyxin B (Mazzei et al., 1982) on NA release induced by Ca^{2+} and/or by PMA. These inhibitors all inhibit PKC-mediated B-50 phosphorylation in SPMs (Dekker et al., 1990*b*) as well as in SL-O-permeated synaptosomes (not shown). Polymyxin B (200 IU/ml) inhibited 10^{-7} *M* PMA-induced NA release by 46%, inhibited the 10^{-5} *M* Ca^{2+}-induced release by 64%, and inhibited the release induced by 10^{-7} *M* PMA at 10^{-5} *M* Ca^{2+} by 56% (Fig. 7). At 2,000 IU/ml, polymyxin B inhibited Ca^{2+}- and PMA-induced release almost completely (Fig. 7). H-7 (10^{-4} *M*) inhibited 10^{-7} *M* PMA-induced release by 57% (Fig. 8). However, H-7 reduced 10^{-5} *M* Ca^{2+}-

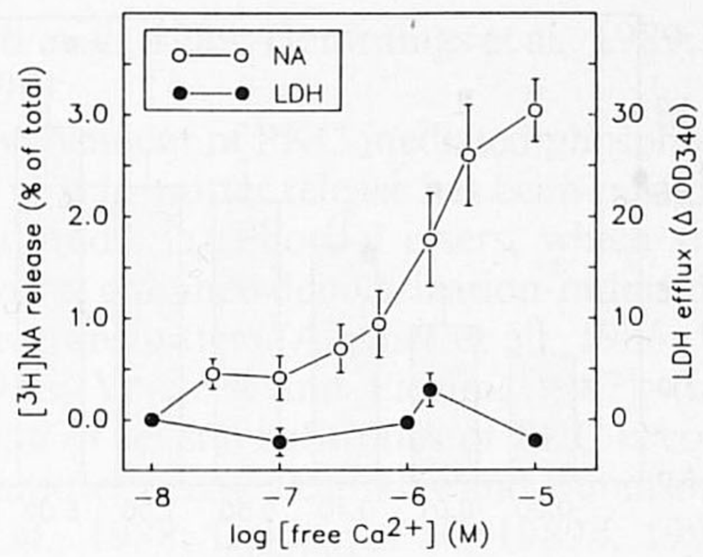

FIG. 3. Ca^{2+} dependency of release of NA and LDH from SL-O-permeated synaptosomes. Synaptosomes were permeated with 0.8 IU/ml SL-O at free Ca^{2+} concentrations as indicated. The permeation buffer contained 5 m*M* ATP. Release of NA or LDH was calculated by subtracting the efflux of NA (LDH) from permeated synaptosomes at 10^{-8} *M* Ca^{2+} from that at the indicated concentration of free Ca^{2+}. Data are means ± SEM of 6–20 observations obtained from six independent experiments.

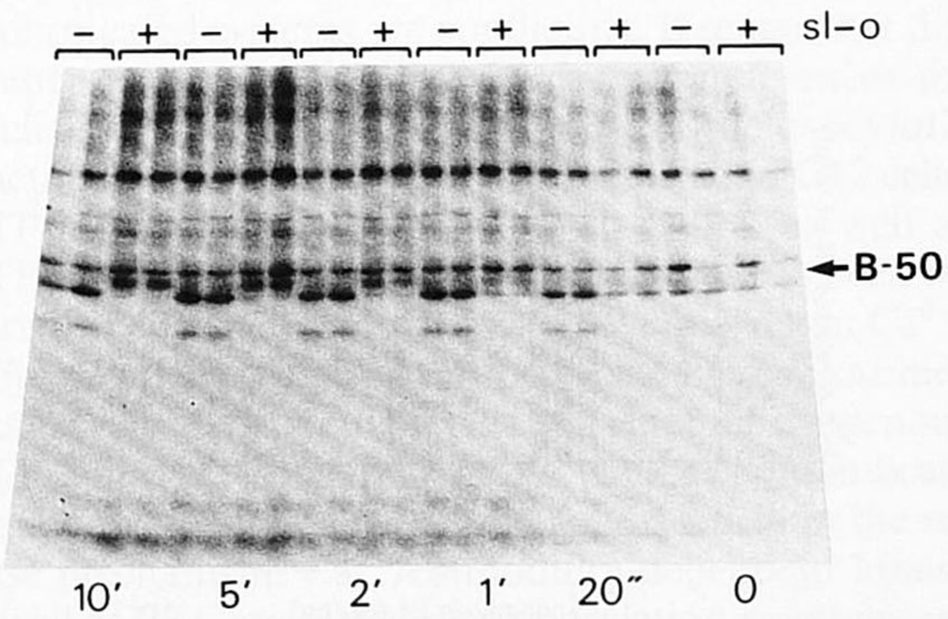

FIG. 2. Autoradiogram showing the time dependency of the effect of SL-O permeation on synaptosomal protein phosphorylation. Synaptosomes were incubated for 0–10 min with (+) or without (−) 0.4 IU/ml SL-O. Phosphorylation was started by the addition of 7.5 μM [γ-^{32}P]ATP and stopped after 15 s by addition of SDS-containing stop mix. Protein phosphorylation was analyzed by SDS-PAGE and autoradiography. Samples were run in duplicate.

TABLE 1. *Effect of changing the cation composition of the permeation buffer*

$[Na^+]$ (m*M*)	$[K^+]$ (m*M*)	Ratio	Efflux at 10^{-7} *M* Ca^{2+} (% of total)	Efflux at 10^{-5} *M* Ca^{2+} (% of total)	Ca^{2+}-dependent release (% of total)
123	5	24.2	26.1 ± 0.3	30.5 ± 0.6	4.4 ± 0.5
21	107	0.2	25.8 ± 0.8	29.8 ± 0.8	4.0 ± 0.8

Synaptosomes were permeated with 0.8 IU/ml SL-O at 10^{-7} and 10^{-5} *M* Ca^{2+} at the indicated concentrations of Na^+ and K^+. Ca^{2+}-dependent release was calculated by subtracting NA efflux at 10^{-7} *M* Ca^{2+} from that at 10^{-5} *M* Ca^{2+} for each condition. Data are means ± SEM of six observations obtained from two independent experiments.

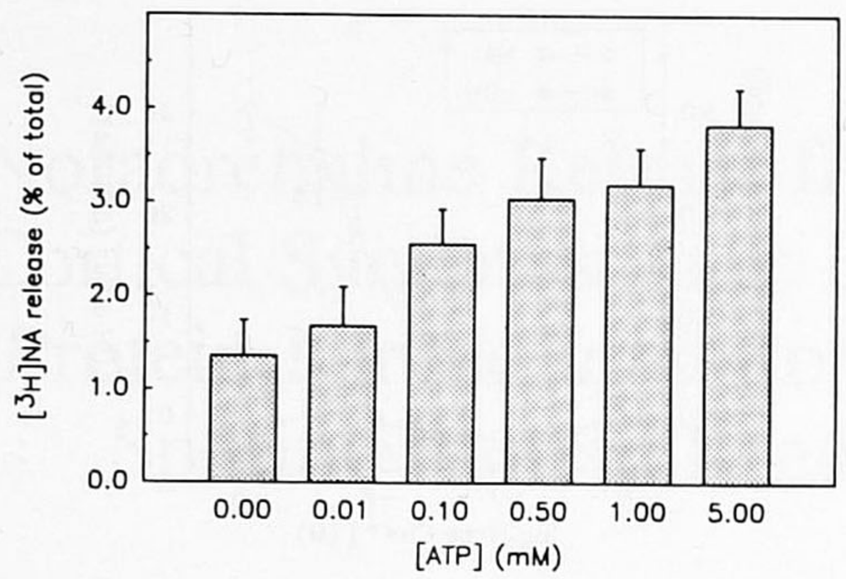

FIG. 4. ATP dependency of release of NA from SL-O-permeated synaptosomes. Synaptosomes were permeated with 0.8 IU/ml SL-O at 10^{-7} and 10^{-5} *M* Ca^{2+} in the presence of 0.00–5.00 m*M* ATP. Release was calculated by subtracting NA efflux at 10^{-7} *M* Ca^{2+} from that at 10^{-5} *M* Ca^{2+} for each concentration of ATP. Data are means ± SEM of 12 observations obtained from four independent experiments.

induced release by only 16% ($p < 0.05$) and the release induced by 10^{-7} *M* PMA at 10^{-5} *M* Ca^{2+} by only 47%, thus reducing release to the level obtained at 10^{-5} *M* Ca^{2+} alone (Fig. 8).

DISCUSSION

In the present article we describe a method to permeate rat brain synaptosomes using the bacterial toxin SL-O. Conditions for efficient permeation were determined by measuring the degree of phosphorylation of B-50 (an intrasynaptosomal PKC substrate) using the membrane-impermeable molecule [γ-^{32}P]ATP and the extent of Ca^{2+}-evoked NA release at different SL-O concentrations. It was established that a 5-min treatment with 0.8 IU/ml SL-O was optimal to permeate synaptosomes without damaging the release machinery. These conditions are comparable with those used by Howell and Gomperts (1987) to permeate rat mast cells with SL-O.

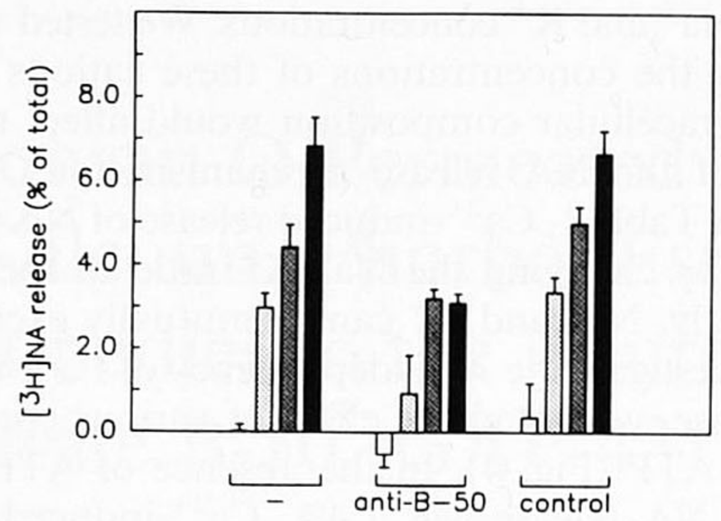

FIG. 6. Effect of antibodies to B-50 on Ca^{2+}- and PMA-induced NA release. Synaptosomes were permeated with 0.8 IU/ml SL-O at 10^{-7} and 10^{-5} *M* Ca^{2+} with or without 10^{-7} *M* PMA. The permeation buffer contained 5 m*M* ATP. B-50 antibodies (anti-B-50) or control antibodies (control) were present at a concentration of 20 μg/assay. NA release was calculated as described in the legend to Fig. 5. Open columns, 10^{-7} *M* Ca^{2+}; widely stippled columns, 10^{-5} *M* Ca^{2+}; densely stippled columns, 10^{-7} *M* Ca^{2+} + 10^{-7} *M* PMA; filled columns, 10^{-5} *M* Ca^{2+} + 10^{-7} *M* PMA. Data are means ± SEM of six observations obtained from two independent experiments.

Under these optimal permeation conditions, Ca^{2+} is a trigger for efflux of NA. Elevation of the free Ca^{2+} concentration from 10^{-8} to 10^{-5} *M* induced a NA release that is quantitatively comparable with the release from intact synaptosomes induced by 10 m*M* K^+ (Dekker et al., 1989*a*). The Ca^{2+} trigger did not affect the SL-O-induced efflux of the cytosolic marker protein LDH. Thus, the Ca^{2+}-induced NA efflux originates from noncytosolic pools of NA and most likely represents vesicular release of NA. The Ca^{2+} sensitivity of NA release from permeated synaptosomes was similar to that observed for catecholamine secretion from permeated PC12 cells and chromaffin cells and to histamine secretion from permeated mast cells (Ahnert-Hilger et al., 1985*a*; Lee and Holz, 1986; Howell and Gomperts, 1987). Free Ca^{2+} levels below 10^{-7} *M* did not induce release in any of the systems, but levels of 10^{-6}–10^{-5} *M* Ca^{2+} were found to be effective. The high

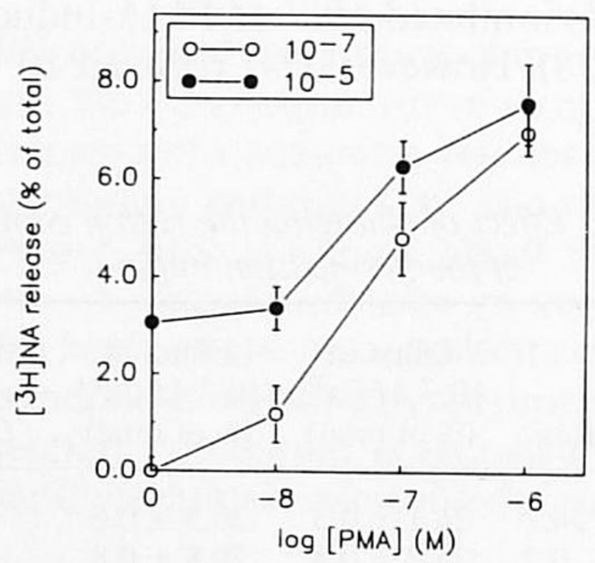

FIG. 5. Effect of PMA on NA release from SL-O-permeated synaptosomes at 10^{-7} *M* and at 10^{-5} *M* Ca^{2+}. Synaptosomes were permeated with 0.8 IU/ml SL-O at 10^{-7} and 10^{-5} *M* Ca^{2+} at different PMA concentrations. The permeation buffer contained 5 m*M* ATP. Release was calculated by subtracting efflux at 10^{-7} *M* Ca^{2+} from efflux at each of the indicated conditions. Data are means ± SEM of six observations obtained from two independent experiments.

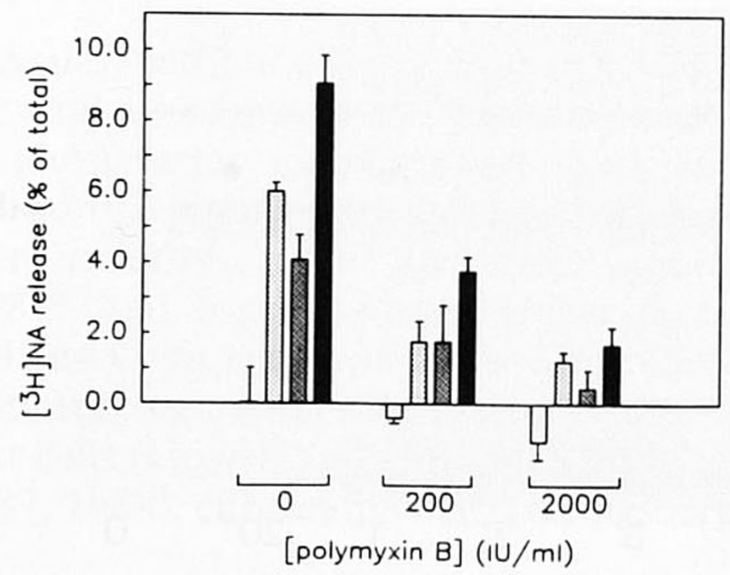

FIG. 7. Effect of polymyxin B on Ca^{2+}- and PMA-induced NA release. Synaptosomes were permeated with 0.8 IU/ml SL-O at 10^{-7} and 10^{-5} *M* Ca^{2+} with or without 10^{-7} *M* PMA. ATP was present at 0.1 m*M*. Polymyxin B was present at a concentration of 200 and 2,000 IU/ml. NA release was calculated as described in the legend to Fig. 5. Columns are described in the legend to Fig. 6. Data are means ± SEM of three observations.

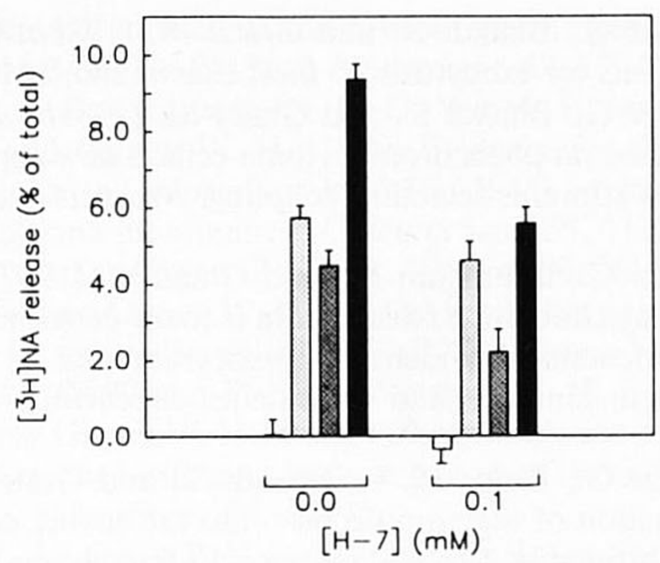

FIG. 8. Effect of H-7 on Ca^{2+}- and PMA-induced NA release. Synaptosomes were permeated with 0.8 IU/ml SL-O at 10^{-7} and 10^{-5} *M* Ca^{2+} with or without 10^{-7} *M* PMA. ATP was present at a concentration of 0.1 m*M*. H-7 was present at a concentration of 10^{-4} *M*. NA release was calculated as described in the legend to Fig. 5. Columns are described in the legend to Fig. 6. Data are means ± SEM of three observations.

Ca^{2+} concentration necessary to induce release contrasts with the overall Ca^{2+} levels measured after K^+ depolarization of intact synaptosomes, which do not exceed the 0.5×10^{-6} *M* level (Verhage et al., 1988). There are two explanations for this apparent contradiction: (a) Local high levels of Ca^{2+} are required to induce transmitter release (Smith and Augustine, 1988), and (b) high concentrations of Ca^{2+} are necessary in the permeated system to overcome SL-O-induced damage to the release machinery or dilution of cytosolic components of the release system.

The Ca^{2+} sensitivity of the NA release system is not affected by the monovalent cation composition of the buffer. Ca^{2+}-dependent release induced at extracellular cation concentrations (high sodium and low potassium) is the same as that induced at more intracellular circumstances (low sodium and high potassium). Apparently the ratio between both ions is not critical for Ca^{2+}-dependent release to occur.

Data on the nucleotide requirement of the release in permeated systems are conflicting. It seems that different secretory cells display varying preferences for nucleoside triphosphates in support of the exocytotic reaction (Howell et al., 1987). In permeated PC12 cells, ATP-independent (Reynolds et al., 1982) as well as ATP-dependent release of catecholamines has been reported (Peppers and Holz, 1986). We found that Ca^{2+}-induced release of NA from permeated synaptosomes was largely dependent on the presence of exogenous ATP. This ATP sensitivity of NA release could indicate the involvement of protein phosphorylation in the release mechanism. Ca^{2+}/calmodulin-dependent kinase as well as PKC-mediated phosphorylation reactions are thought to play a role in transmitter release. The first system is thought to affect release via the neuron-specific vesicle-associated protein synapsin I. This protein is thought to be involved in vesicle-cytoskeletal interactions and its phosphorylation probably liberates exocytotic vesicles from their cytoskeletal environment (Benfenati et al., 1989; Hemmings et al., 1989; Nichols et al., 1990).

The involvement of PKC-mediated phosphorylation reactions in transmitter release has been postulated on several grounds: (a) Phorbol esters, which stimulate PKC in vitro, enhance depolarization-induced release of various transmitters (Allgaier et al., 1986; Wakade et al., 1986; Versteeg and Florijn, 1987); (b) phosphorylation of several substrates of PKC is correlated with transmitter release (Dunkley and Robinson, 1986; Wang et al., 1988; Dekker et al., 1989*b*, 1990*a*); (c) antibodies to the PKC substrate B-50, interfering with the phosphorylation of B-50, inhibit Ca^{2+}-induced release of NA from permeated synaptosomes (this study; Dekker et al., 1989*a*); (d) polymyxin B, known to inhibit PKC and PKC-mediated B-50 phosphorylation, completely abolishes depolarization-induced release (Allgaier and Hertting, 1986; Versteeg and Ulenkate, 1987; Dekker et al., 1990*b*) (the fact that other inhibitors of PKC appear to be less effective is discussed below); and (e) prolonged phorbol ester treatment of PC12 cells resulted in a down-regulation of PKC and a concomitant decrease in depolarization-induced release of NA (Matthies et al., 1987).

In this study we show that phorbol esters stimulate NA release from permeated synaptosomes even at 10^{-8} *M* Ca^{2+}. As the effect of the phorbol esters is stereospecific, PKC seems to be the target for the phorbol esters. Only at high concentrations ($>10^{-7}$ *M*) are PMA- and Ca^{2+}-induced NA release not additive (Fig. 6). Our interpretation of these data is that the effect of the two release stimuli is additive and that the convergence at high PMA concentrations arises from an overstimulation of the release system, possibly due to excessive translocation of PKC from the cytosol to the membrane or nonspecific effects of PMA. During excessive stimulation the amount of vesicles available for release could be a limiting factor. If our interpretation that Ca^{2+}- and PMA-induced release are additive is correct, this implies that they stimulate NA release through different mechanisms.

Thus, we compared the sensitivity of Ca^{2+}- and PMA-induced release to the protein kinase inhibitors polymyxin B (Mazzei et al., 1982) and H-7 (Hidaka et al., 1984) and to B-50 antibodies. All three inhibit PKC-mediated B-50 phosphorylation in permeated synaptosomes, showing that they indeed interfere with the PKC system. Ca^{2+}-induced release can be inhibited by antibodies to B-50 and by polymyxin B, but not by H-7. PMA-induced release can be inhibited by polymyxin B and H-7, but only marginally by B-50 antibodies. Our effects of polymyxin B and H-7 are in agreement with those obtained by others in intact synaptosomes and hippocampal slices. In both preparations, polymyxin B completely inhibits evoked release of various transmitters (Allgaier and Hertting, 1986; Versteeg and Ulenkate, 1987) and H-7 has only marginal effects (Daschmann et al., 1988), whereas phorbol ester-induced transmitter release could be inhibited by all PKC

inhibitors tested by these authors. Thus, evoked and Ca^{2+}-induced release are not affected by the most specific PKC inhibitor, H-7, whereas phorbol ester-induced release is completely blocked. These data are in line with the hypothesis that Ca^{2+} and phorbol ester induce release through different mechanisms. This hypothesis is further supported by the finding that B-50 antibodies inhibit Ca^{2+}- but not PMA-induced release.

The precise mechanisms of Ca^{2+}- and PMA-induced release and their relationship remain to be elucidated. PMA-induced release probably involves translocation of PKC to the membrane, but it is not clear to what extent PKC translocation contributes to evoked NA release under physiological conditions. Although our studies clearly show an involvement of PKC substrate B-50 in Ca^{2+}-induced NA release from permeated synaptosomes, they do not provide conclusive evidence for a role of PKC-mediated B-50 phosphorylation in release. Our results leave open the possibility that other properties of B-50, which are affected by the B-50 antibodies or polymyxin B, also contribute to the release process. An interesting possibility is the calmodulin-binding property of B-50. It has been shown that purified B-50 has a higher affinity for calmodulin in the absence of Ca^{2+} than in the presence (Andreasen et al., 1983; Cimler et al., 1987) and that prephosphorylation of purified B-50 by PKC inhibits calmodulin binding (Alexander et al., 1987, 1988). Based on these in vitro studies, it has been suggested (Alexander et al., 1987) that in vivo B-50 may act as a local calmodulin store. Indeed, we have recently provided evidence for the binding of CaM to endogenous B-50 in native SPMs (De Graan et al., 1990). Calmodulin may be dissociated from B-50 after a depolarization-induced rise in intracellular Ca^{2+} and subsequently activate cellular processes involved in transmitter release, for instance, calmodulin-dependent kinases and phosphorylation of synapsin I (Nichols et al., 1990). Once calmodulin and B-50 have been dissociated, B-50 may be phosphorylated by PKC, thus preventing reassociation of calmodulin and prolonging calmodulin action. Although this hypothesis needs confirmation in physiological systems, it provides an exciting link between PKC and calmodulin-dependent processes implicated in the mechanism of neurotransmitter release. Therefore, we will focus our research on the role of B-50 in transmitter release on the interaction between the different molecular properties of the B-50 protein.

Acknowledgment: We gratefully acknowledge the experimental assistance of Wiep Scheper, Marina de Wit, and Ank Frankhuyzen. We thank Dr. D. H. G. Versteeg for helpful advice and Ed Kluis for the artwork. L.V.D. has been supported by Medigon (grant 900-548-082).

REFERENCES

Ahnert-Hilger G. and Gratzl M. (1987) Further characterization of dopamine release by permeabilized PC12 cells. *J. Neurochem.* **49,** 764–770.

Ahnert-Hilger G., Bhakdi S., and Gratzl M. (1985*a*) Minimal requirements for exocytosis. *J. Biol. Chem.* **260,** 12730–12734.

Ahnert-Hilger G., Bhakdi S., and Gratzl M. (1985*b*) α-Toxin permeabilized rat pheochromocytoma cells: a new approach to investigate stimulus-secretion coupling. *Neurosci. Lett.* **58,** 107–110.

Ahnert-Hilger G., Bräutigam M., and Gratzl M. (1987) Ca^{2+}-stimulated catecholamine release from α-toxin-permeabilized PC12 cells: biochemical evidence for exocytosis and its modulation by protein kinase C and G proteins. *Biochemistry* **26,** 7842–7848.

Ahnert-Hilger G., Bader M. F., Bhakdi S., and Gratzl M. (1989) Introduction of macromolecules into rat bovine adrenal medullary chromaffin cells and rat pheochromocytoma cells (PC12) by permeabilization with streptolysin O: inhibitory effect of tetanus toxin on catecholamine secretion. *J. Neurochem.* **52,** 1751–1758.

Alexander K. A., Cimler B. M., Meier K. E., and Storm D. R. (1987) Regulation of calmodulin binding to P-57. *J. Biol. Chem.* **262,** 6108–6113.

Alexander K. A., Wakim B. T., Doyle G. S., Walsh K. A., and Storm D. R. (1988) Identification and characterization of the calmodulin-binding domain of neuromodulin, a neuron-specific calmodulin-binding protein. *J. Biol. Chem.* **263,** 7544–7549.

Allgaier C. and Hertting G. (1986) Polymyxin B, a selective inhibitor of protein kinase C, diminishes the release of noradrenaline and the enhancement of release caused by phorbol 12,13-dibutyrate. *Naunyn Schmiedebergs Arch. Pharmacol.* **334,** 218–221.

Allgaier C., Von Kügelgen O., and Hertting G. (1986) Enhancement of noradrenaline release by 12-*O*-tetradecanoyl phorbol-13-acetate, an activator of protein kinase C. *Eur. J. Pharmacol.* **129,** 389–392.

Aloyo V. J., Zwiers H., and Gispen W. H. (1983) Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase. *J. Neurochem.* **41,** 649–653.

Andreasen T. J., Luetje C. W., Heideman W., and Storm D. R. (1983) Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry* **22,** 4615–4618.

Basi G. S., Jacobson R. D., Virag I., Schilling J., and Skene J. H. P. (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. *Cell* **49,** 785–791.

Benfenati F., Bähler M., Jahn R., and Greengard P. (1989) Interactions of synapsin I with small synaptic vesicles: distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins. *J. Cell Biol.* **108,** 1863–1872.

Bergmeyer H. U. and Bernt E. (1970) Lactat-dehydrogenase; UV-test mit pyruvat und NADH, in *Methoden der Enzymatischen Analyse* (Bergmeyer H. U., ed), pp. 533–538. Verlag Chemie, Weinheim.

Bittner M. A. and Holz R. W. (1988) Effects of tetanus toxin on catecholamine release from intact and digitonin-permeabilized chromaffin cells. *J. Neurochem.* **51,** 451–456.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **76,** 248–254.

Cimler B. M., Giebelhaus D. H., Wakim B. T., Storm D. R., and Moon R. T. (1987) Characterization of murine cDNAs encoding P-57, a neural-specific calmodulin-binding protein. *J. Biol. Chem.* **262,** 12158–12163.

Daschmann B., Allgaier C., Nakov R., and Hertting G. (1988) Staurosporine counteracts the phorbol ester-induced enhancement of neurotransmitter release in hippocampus. *Arch. Int. Pharmacodyn. Ther.* **296,** 232–245.

De Graan P. N. E., Heemskerk F. M. J., Dekker L. V., Melchers B. P. C., Gianotti C., and Schrama L. H. (1988) Phorbol esters induce long- and short-term enhancement of B-50/GAP-43 phosphorylation in rat hippocampal slices. *Neurosci. Res. Commun.* **3,** 175–182.

De Graan P. N. E., Oestreicher A. B., Dekker L. V., Van der Voorn L., and Gispen W. H. (1989) Determination of changes in the

phosphorylation state of the neuron specific protein kinase C substrate B-50 (GAP-43). *J. Neurochem.* **52,** 17–23.

De Graan P. N. E., Oestreicher A. B., De Wit M., Kroef M., Schrama L. H., and Gispen W. H. (1990) Evidence for the binding of calmodulin to endogenous B-50 (GAP-43) in native synaptosomal plasma membranes. *J. Neurochem.* **55,** 2139–2141.

Dekker L. V., De Graan P. N. E., Oestreicher A. B., Versteeg D. H. G., and Gispen W. H. (1989*a*) Inhibition of noradrenaline release by antibodies to B-50 (GAP-43). *Nature* **342,** 74–76.

Dekker L. V., De Graan P. N. E., Versteeg D. H. G., Oestreicher A. B., and Gispen W. H. (1989*b*) Phosphorylation of B-50 (GAP-43) is correlated with neurotransmitter release in rat hippocampal slices. *J. Neurochem.* **52,** 24–30.

Dekker L. V., De Graan P. N. E., Spierenburg H., De Wit M., Versteeg D. H. G., and Gispen W. H. (1990*a*) Evidence for a relationship between B-50 and [^{3}H]-noradrenaline release in rat brain synaptosomes. *Eur. J. Pharmacol.* **188,** 113–122.

Dekker L. V., De Graan P. N. E., De Wit M., Hens J. J. H., and Gispen W. H. (1990*b*) Depolarization-induced phosphorylation of the protein kinase C substrate B-50/GAP-43 in rat cortical synaptosomes. *J. Neurochem.* **54,** 1645–1652.

De Langen C. J., Hogeboom F., and Mulder A. (1979) Presynaptic noradrenergic α-receptors and modulation of [^{3}H]noradrenaline release from rat brain synaptosomes. *Eur. J. Pharmacol.* **60,** 79–89.

Dunkley P. R. and Robinson P. J. (1986) Depolarization-dependent protein phosphorylation in synaptosomes: mechanisms and significance. *Progr. Brain Res.* **69,** 273–294.

Gispen W. H., Leunissen J. L. M., Oestreicher A. B., Verkleij A. J., and Zwiers H. (1985) Presynaptic localization of B-50 phosphoprotein: the (ACTH)-sensitive protein kinase substrate involved in rat brain polyphosphoinositide metabolism. *Brain Res.* **328,** 381–385.

Hemmings H. C., Nairn A. C., McGuinness T. L., Huganir R. L., and Greengard P. (1989) Role of protein phosphorylation in neuronal signal transduction. *FASEB J.* **3,** 1583–1592.

Hidaka H., Inagaki M., Kawamoto S., and Sasaki Y. (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23,** 5036–5041.

Howell T. W. and Gomperts B. D. (1987) Rat mast cells permeabilized with streptolysin O secrete histamine in response to Ca^{2+} at concentrations buffered in the micromolar range. *Biochim. Biophys. Acta* **927,** 177–183.

Howell T. W., Cockcroft S., and Gomperts B. D. (1987) Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. *J. Cell Biol.* **105,** 191–197.

Howell T. W., Kramer Y. M., and Gomperts B. D. (1989) Protein phosphorylation and the dependence on Ca^{2+} and GTP-γ-S for exocytosis from permeabilized mast cells. *Cell. Signal* **1,** 157–163.

Karns L. R., Ng S.-C., Freeman J. A., and Fishman M. C. (1987) Cloning of complementary DNA for GAP-43, a neuronal growth-related protein. *Science* **236,** 597–600.

Kenigsberg R. L. and Trifaro J. M. (1985) Micro-injection of calmodulin antibodies into cultured chromaffin cells blocks catecholamine release in response to stimulation. *Neuroscience* **14,** 335–347.

Knight D. E. and Scrutton M. C. (1986) Gaining access to the cytosol: the technique and some applications of electro-permeabilization. *Biochem. J.* **234,** 497–506.

Kristjansson G. I., Zwiers H., Oestreicher A. B., and Gispen W. H. (1982) Evidence that the synaptic phosphoprotein B-50 is localized exclusively in nerve tissue. *J. Neurochem.* **39,** 371–378.

Lee S. A. and Holz R. W. (1986) Protein phosphorylation and secretion in digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.* **261,** 17089–17098.

Matthies H. J. G., Palfrey H. C., Hirning L. D., and Miller R. J. (1987) Down regulation of protein kinase C in neuronal cells: effects on neurotransmitter release. *J. Neurosci.* **7,** 1198–1206.

Mazzei G. J., Katoh N., and Kuo J. F. (1982) Polymyxin B is a more selective inhibitor for phospholipid-sensitive Ca^{2+}-dependent protein kinase than for calmodulin-sensitive Ca^{2+}-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **109,** 1129–1133.

Nichols R. A., Wu W. C. S., Haycock J. W., and Greengard P. (1989) Introduction of impermeant molecules into synaptosomes using freeze/thaw permeabilization. *J. Neurochem.* **52,** 521–529.

Nichols R. A., Sihra T. S., Czernik A. J., Nairn A., and Greengard P. (1990) Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. *Nature* **343,** 647–651.

Nielander H. B., Schrama L. H., Van Rozen A. J., Kasperaitis M., Oestreicher A. B., De Graan P. N. E., Gispen W. H., and Schotman P. (1987) Primary structure of the neuron-specific phosphoprotein B-50 is identical to growth-associated protein GAP-43. *Neurosci. Res. Commun.* **1,** 163–172.

Oestreicher A. B., Van Dongen C. J., Zwiers H., and Gispen W. H. (1983) Affinity-purified anti-B-50 protein antibody: interference with the function of phosphoprotein B-50 in synaptic plasma membranes. *J. Neurochem.* **41,** 331–340.

Peppers S. C. and Holz R. W. (1986) Catecholamine secretion from digitonin-treated PC12 cells. *J. Biol. Chem.* **261,** 14665–14669.

Reynolds E. E., Melega W. P., and Howard B. D. (1982) Adenosine 5′-triphosphate independent secretion from PC12 pheochromocytoma cells. *Biochemistry* **21,** 4795–4799.

Rosenthal A., Chan S. Y., Henzel W., Haskell C., Kuang W.-J., Chen E., Wilcox J. N., Ullrich A., Goeddel D. V., and Routtenberg A. (1987) Primary structure and mRNA localization of protein F1, a growth-related protein kinase C substrate associated with synaptic plasticity. *EMBO J.* **6,** 3641–3646.

Schweizer F. E., Schäfer T., Tapparelli C., Grob M., Karli U. O., Heumann R., Thoenen H., Bookman R. J., and Burger M. M. (1989) Inhibition of exocytosis by intracellularly applied antibodies against a chromaffin granule-binding protein. *Nature* **339,** 709–712.

Smith S. J. and Augustine G. J. (1988) Calcium ions, active zones and synaptic transmitter release. *Trends Neurosci.* **11,** 458–464.

Sörensen R. G., Kleine L. P., and Mahler H. R. (1981) Presynaptic localization of phosphoprotein B-50. *Brain Res. Bull.* **7,** 57–61.

Van Lookeren Campagne M., Oestreicher A. B., Van Bergen en Henegouwen P. M. P., and Gispen W. H. (1989) Ultrastructural immunocytochemical localization of B-50/GAP-43, a protein kinase C substrate, in isolated presynaptic nerve terminals and neuronal growth cones. *J. Neurocytol.* **18,** 479–489.

Verhage M., Besselsen E., Lopes da Silva F. H., and Ghijsen W. E. J. M. (1988) Evaluation of the Ca^{2+} concentration in purified nerve terminals: relationship between Ca^{2+} homeostasis and synaptosomal preparation. *J. Neurochem.* **51,** 1667–1674.

Versteeg D. H. G. and Florijn W. J. (1987) Phorbol 12,13-dibutyrate enhances electrically stimulated neuromessenger release from rat dorsal hippocampal slices in vitro. *Life Sci.* **40,** 1237–1243.

Versteeg D. H. G. and Ulenkate H. J. L. M. (1987) Basal and electrically stimulated release of [^{3}H]-noradrenaline and [^{3}H]-dopamine from rat amygdala slices in vitro: effects of 4β-phorbol 12,13-dibutyrate, 4α-phorbol 12,13-didecanoate and polymyxin B. *Brain Res.* **416,** 343–348.

Wakade A. R., Malhotra R. K., and Wakade T. D. (1986) Phorbol ester, an activator of protein kinase C, enhances calcium-dependent release of sympathetic neurotransmitter. *Naunyn Schmiedebergs Arch. Pharmacol.* **331,** 122–124.

Wang J. K. T., Walaas S. I., and Greengard P. (1988) Protein phosphorylation in nerve terminals: comparison of calcium/calmodulin-dependent and calmodulin/diacylglycerol-dependent systems. *J. Neurosci.* **8,** 281–288.

Zwiers H., Schotman P., and Gispen W. H. (1980) Purification and some characteristics of an ACTH-sensitive protein kinase and its substrate protein in rat brain membranes. *J. Neurochem.* **34,** 1689–1699.