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European Journal of Pharmacology
Molecular Pharmacology Section 291 (1995) 387–398

ejp
molecular pharmacology

Ba^{2+} replaces Ca^{2+}/calmodulin in the activation of protein phosphatases and in exocytosis of all major transmitters

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Received 8 June 1995; revised 16 August 1995; accepted 22 August 1995

Abstract

Exocytosis from nerve terminals is triggered by depolarization-evoked Ca^{2+} entry, which also activates calmodulin and stimulates protein phosphorylation. Ba^{2+} is believed to replace Ca^{2+} in triggering exocytosis without activation of calmodulin and can therefore be used to unravel aspects of presynaptic function. We have analysed the cellular actions of Ba^{2+} in relation to its effect on transmitter release from isolated nerve terminals. Barium evoked specific release of amino acid transmitters, catecholamines and neuropeptides (EC_{50} 0.2–0.5 mM), similar to K^+/Ca^{2+}-evoked release both in extent and kinetics. Ba^{2+}- and Ca^{2+}-evoked release were not additive. In contrast to Ca^{2+}, Ba^{2+} triggered release which was insensitive to trifluoperizine and hardly stimulated protein phosphorylation. These observations are in accordance with the ability of Ba^{2+} to replace Ca^{2+} in exocytosis without activating calmodulin. Nevertheless, calmodulin appears to be essential for regular (Ca^{2+}-triggered) exocytosis, given its sensitivity to trifluoperizine. Both Ba^{2+}- and Ca^{2+}-evoked release were blocked by okadaic acid. Furthermore, anti-calcineurin antibodies decreased Ba^{2+}-evoked release. In conclusion, Ba^{2+} replaces Ca^{2+}/calmodulin in the release of the same transmitter pool. Calmodulin-dependent phosphorylation appears not to be essential for transmitter release. Instead, our data implicate both Ca^{2+}-dependent and -independent *de*phosphorylation in the events prior to neurotransmitter exocytosis.

Keywords: Exocytosis; Ba^{2+}; Ca^{2+}-dependent release; Calmodulin; Okadaic acid; Phosphatase

1. Introduction

The regulated exocytosis of neurotransmitters and neuropeptides is triggered by depolarization evoked Ca^{2+} entry. Although this process is reasonably specific for Ca^{2+}, several divalent cations with similar physico-chemical properties, such as Ba^{2+}, Sr^{2+} and Pb^{2+}, were found to evoke transmitter release as well (Zengel and Magleby, 1980; Augustine and Eckert, 1984; Heldman et al., 1989; Shao and Suszkiw, 1991; McMahon and Nicholls, 1993; Sihra et al., 1993). Among these, Ba^{2+} is the best documented and probably the most effective. However, Ba^{2+} was found to be ineffective in binding and activating the Ca^{2+} binding protein calmodulin (Chao et al., 1984; Klee, 1988), although activation of calmodulin and the Ca^{2+}/calmodulin-dependent kinase II, the autophosphorylation of this kinase and the phosphorylation of its major substrate in the presynaptic terminal, synapsin I, have all been implicated in the presynaptic stimulus-secretion coupling (see for a review Greengard et al., 1993). Hence, if Ba^{2+}-evoked release is true vesicular release, this contradicts the suggested role of calmodulin-dependent processes. One important indication that Ba^{2+} can indeed trigger true vesicular release came from the use of clostridial toxins that specifically cleave components of the docking-fusion complex of synaptic vesicles and the plasma membrane. We have previously shown that Ca^{2+}- and Ba^{2+}-evoked release are equally sensitive to these toxins (McMahon et al., 1992). For these reasons Ba^{2+} may be

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SSDI 0922-4106(95)00132-8

used to unravel aspects of presynaptic function and to pinpoint which of the many cellular actions of Ca^{2+} are relevant to the mechanism of transmitter release.

Ba^{2+} may influence transmitter release at different stages within the nerve terminal. Ba^{2+} may depolarize the terminal by its unbalanced influx and by its inhibitory effect on K^+ channels (see Augustine and Eckert, 1984; Mironov and Juri, 1990; McMahon and Nicholls, 1993; Sihra et al., 1993). Intracellularly, Ba^{2+} may exchange with Ca^{2+} in internal stores. Finally, Ba^{2+} may interact with factors other than calmodulin to trigger transmitter release.

In the present study we analyse the cellular actions of Ba^{2+} in isolated nerve terminals in relation to its ability to evoke transmitter release. This analysis focuses on three questions. Firstly, is Ba^{2+}-evoked release true exocytotic (vesicular) release, indistinguishable from Ca^{2+}-evoked release? Secondly, is transmitter release indeed independent of calmodulin activation and calmodulin-dependent protein phosphorylation? Thirdly, which cellular actions of Ba^{2+}, if not calmodulin activation and calmodulin-dependent protein phosphorylation, are responsible for its stimulatory effect on transmitter release? Assay systems have been established to discriminate between indirect effects of Ba^{2+} on the membrane potential, on intracellular Ca^{2+} stores and on non-vesicular transmitter pools by using tetrodotoxin to reduce the instability of the membrane potential evoked by Ba^{2+} (see McMahon and Nicholls, 1993; Sihra et al., 1993), by investigating additivity between Ba^{2+}-evoked release and K^+/Ca^{2+}-evoked release and by exploiting both intact and permeated nerve terminals. In order to investigate the discrepancy between Ba^{2+}-evoked transmitter release and the role of calmodulin in the same process, we have characterized the effects of the calmodulin inhibitor trifluoperizine on Ba^{2+}- and Ca^{2+}-evoked release. In addition, the role of both ions in regulating protein phosphorylation and dephosphorylation was studied. Because different classes of transmitter appear to be regulated by different Ca^{2+} concentrations and are released from different loci within the nerve terminal (Verhage et al., 1991b), we have also compared representatives of all major classes of transmitter, i.e., the fast-acting amino acid transmitters which are released by high local Ca^{2+} elevations from small synaptic vesicles at the active zone, neuropeptides which are released by small Ca^{2+} elevations at ectopic sites within the terminal and catecholamines which may utilise both secretion pathways (see for a review Verhage et al., 1995).

2. Materials and methods

2.1. Materials

Percoll was obtained from Pharmacia (Uppsala, Sweden); bacitracin, trifluoperizine, calmodulin and calcineurin from Sigma (St. Louis, MO, USA); Ionomycin from Calbiochem (La Jolla, CA, USA); ATP from Boehringer (Mannheim, Germany); streptolysin-O from Murex (Utrecht, Netherlands); fura-2-AM from Molecular Probes (Eugene OR, USA); okadaic acid from Anawa (Wangen, Switzerland) and [γ-^{32}P]ATP (3000 Ci/mmol) and [7,8-^{3}H]noradrenaline (34 Ci/mmol) from Amersham (Buckinghamshire, UK). All other chemicals, of the purest grade available, were obtained from Merck (Darmstadt, Germany). Fura-2-AM, ionomycin, trifluoperizine and okadaic acid were dissolved in dimethyl sulfoxide (2 mM, 1 mM, 10 mM and 5 mM stock, respectively); all other chemicals were dissolved in water.

2.2. Synaptosomal preparation

Synaptosomes were prepared from the whole forebrain of male Wistar rats (180–240 g). Preparations were made with a modification of the methods of Nagy and Delgado-Escueta (1984) and Dunkley et al. (1986) as described before (Verhage et al., 1989), with a 7.5/10/23% discontinuous Percoll/0.32 M sucrose gradients. Synaptosomes were collected from the interface 10–23% and diluted in artificial cerebrospinal fluid (CSF) of the following composition: 3 mM KCl, 122 mM NaCl, 1.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 20 mM NaTES, 10 mM glucose, 50 μM EGTA, 100 μM pargyline, 1 mg/ml bacitracin and 1 mg/ml albumin; pH 7.4 using NaOH/HCl. Protein content was measured according to Bradford (1976) with bovine serum albumin as a standard.

2.3. Transmitter release

Synaptosomes (0.75 mg) were preincubated in CSF (0.75 ml) for 5–10 min at 37°C prior to addition of either 15–30 mM KCl and 1.5 mM $CaCl_2$ or 0.2–2 mM $BaCl_2$. Ca^{2+}- and Ba^{2+}-evoked release were estimated by subtracting release in basal medium from release in media containing K^+/Ca^{2+} and/or Ba^{2+}. Since Ba^{2+} may inhibit certain K^+ permeabilities and is believed to produce an unstable, tetrodotoxin-sensitive membrane potential in synaptosomes (McMahon and Nicholls, 1993), 5 μM tetrodotoxin was present in all release experiments. Tetrodotoxin did not affect K^+/Ca^{2+}-evoked release or K^+-evoked $[Ca]_i$ elevations. For estimations of initial release in the first 15 s, incubations were terminated by addition of medium containing 10 mM EGTA. For estimations of total release over 180 s EGTA was omitted. Synaptosomes were separated from the medium by spinning through silicone oil (Kauppinen et al., 1988). Supernatants were generally divided into three aliquots which were (a) stored in liquid nitrogen for catecholamine analysis, (b) mixed with methanol for neuropeptide analysis or (c) mixed with 0.1% (w/v) trichloroacetic acid and 5 μM homoserine (final concentrations) for amino acid analysis. Synaptosomal pellets were washed and sonicated in ice-

cold CSF and then analyzed as described for the supernatants. In some experiments, a multiple batch of synaptosomes was preincubated for 30 min in the presence of okadaic acid and 1.2 mM NaH_2PO_4. Subsequently synaptosomes were spun down, resuspended, divided in single batches and stimulated as described above. Synaptosomes were used within 4 h after completing the preparation.

Catecholamines were extracted, derivatized and analyzed by HPLC using fluorimetric detection (Verhage et al., 1992a); CCK-8 and [Met^5]enkephalin were analyzed by radioimmunoassay (Verhage et al., 1991a, 1992b). The antiserum L48 for CCK-8 analysis was a kind gift of Dr. G.J. Dockray. Amino acids were analyzed using high performance liquid chromatography with fluorimetric detection after *o*-phthaldialdehyde derivatization (Verhage et al., 1989).

For release studies in permeated nerve terminals, synaptosomes were labelled with [^{3}H]noradrenaline and analysed as described (Hens et al., 1993). Synaptosomes (10 μg protein) were permeated with 0.3 IU/ml streptolysin-O as described (Hens et al., 1993). [^{3}H]Noradrenaline release was evoked by adding Ba^{2+} either simultaneously with streptolysin-O at $t = 0$, or after 2.5 min. For calcineurin inhibition, anti-calcineurin antibodies (Momayezi et al., 1987) were present throughout the experiment. In control experiments total rabbit IgGs (Miles, UK) were used. Ba^{2+}-evoked release was calculated by subtracting basal release (in the absence of Ba^{2+}) from the release in the presence of 1 mM Ba^{2+}.

2.4. Determination of the intracellular free Ca^{2+} concentration

Synaptosomes were preincubated in CSF (see above) with 5 μM fura-2-acetoxymethyl ester for 30 min at 37°C as previously described (Verhage et al., 1989). After washing and resuspension in a stirred, thermostat cuvette within a Perkin-Elmer LS5B spectrofluorimeter, synaptosomes were preincubated for a further 10 min at 37°C after which additions were made as detailed in the legends. Data points were collected at 7.5 s intervals, processed on an IBM-compatible PC and $[Ca^{2+}]_i$ calculated as described by Grynkiewicz et al. (1985).

2.5. Protein phosphorylation / dephosphorylation

Synaptosomal plasma membranes were prepared from rat whole forebrain as described earlier (Kristjansson et al., 1982). Aliquots (10 μg in 25 μl) were preincubated for 5 min at 30°C in 10 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA and calmodulin (0.2 IU/μl). For stimulated conditions, either 1 mM Ba^{2+} or 0.2 mM Ca^{2+} was present throughout the experiment. Proteins were phosphorylated with [γ-^{32}P]ATP (7.5 μM, 1 μCi) as described earlier (Dekker et al., 1991; Hens et al., 1993). Proteins were separated by 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorylation was quantified using a Bas1000 Fuji Phosphoimager (Raytest, Germany).

Calcineurin activity was monitored by dephosphorylation of the calcineurin substrate B-50 in cell-free experiments. Rat B-50 (1.0 μg protein isolated according to Zwiers et al. (1986) and purified by chromatography on calmodulin-sepharose; Andreasen et al., 1983; Schrama et al., 1989) was phosphorylated with protein kinase C (PKC) (0.02 μg, purified according to Kikkawa et al., 1986) and 0.4 μg phosphatidylserine in 10 mM Tris/HCl, 10 mM $MgCl_2$ and 0.1 mM $CaCl_2$, pH 7.4 for 20 min and stopped by boiling. Dephosphorylation of B-50 was assayed with 0.5 IU calcineurin in the presence of 5 IU calmodulin and either 0.1 mM Ca^{2+} or 1 mM Ba^{2+} for 20 min at 37°C. Dephosphorylation was quantified by B-50 gel electrophoresis and densitometry (Kristjansson et al., 1982).

3. Results

3.1. Characterization of the Ba^{2+}-evoked release

In isolated nerve terminals, addition of Ba^{2+} evoked the release of all neurotransmitters studied in the absence of extracellular Ca^{2+} and depolarizing agents (in the presence of 3.5 mM K^+ and 50 μM EGTA in the medium). Fig. 1 shows a dose-response curve of the Ba^{2+}-evoked release. Ba^{2+} evoked the specific release of endogenous amino acid transmitters, glutamate and γ-aminobutyric acid (GABA), 4- to 5-fold over basal levels (basal efflux in the absence of extracellular Ba^{2+} or Ca^{2+}, Fig. 1A). This increase was similar in extent to the K^+-evoked, Ca^{2+}-dependent release previously found under similar conditions (Verhage et al., 1989). The half-maximal stimulation was around 0.2 mM Ba^{2+} for both transmitters (Fig. 1A). Ba^{2+} did not produce significant release of other, non-transmitter amino acids, such as tyrosine, threonine, citrulline, alanine and histidine (not shown). Ba^{2+} also evoked the specific release of endogenous catecholamine transmitters, noradrenaline and dopamine, 2.5-fold over basal levels (Fig. 1B). Again, this increase was similar to the K^+-evoked, Ca^{2+}-dependent release previously found under similar conditions (Verhage et al., 1992a). The half-maximal stimulation was between 0.2–0.5 mM Ba^{2+} (Fig. 1B). Ba^{2+} did not evoke significant release of other catecholamines such as adrenaline and dopac, which are not considered to be released by regulated exocytosis within the brain (not shown). Furthermore, Ba^{2+} evoked the release of the two most abundant neuropeptides in brain, cholecystokinin-8 (CCK-8) and [Met^5]enkephalin, 2.5- to 3-fold over basal levels (Fig. 1C). The half-maximal stimulation was between 0.2–0.5 mM Ba^{2+} for both neuropeptides (Fig. 1C). Again, this increase was similar to the K^+-evoked, Ca^{2+}-dependent release previously found under

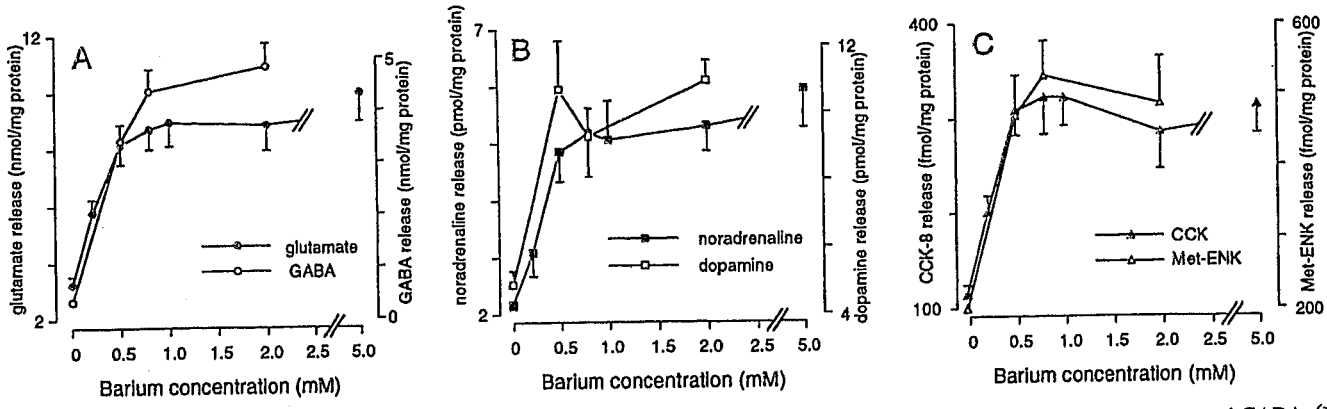

Fig. 1. Dose-response relations for Ba^{2+}-evoked release of different endogenous transmitters. (A) The amino acid transmitters, glutamate and GABA. (B) The catecholamine transmitters, noradrenaline and dopamine. (C) The neuropeptides, cholecystokinin-8 (CCK-8) and [Met^5]enkephalin (Met-ENK). Release was determined after 5 min preincubation followed by 5 min incubation with the indicated Ba^{2+} concentration. The release value at zero Ba^{2+} represents basal efflux and non-specific release during the 5 min pre-incubation period and 5 min further incubation without Ba^{2+} addition (see Materials and methods). Data represent means ± S.E.M. of 4–5 independent preparations. The Ba^{2+}-evoked release is significantly higher than basal efflux (zero Ba^{2+}) for all Ba^{2+} concentrations and all transmitters ($P < 0.01$) except in the case of noradrenaline release at 0.2 mM Ba^{2+} (Fig. B, $P < 0.05$).

similar conditions (Verhage et al., 1991a, 1992b), although Ba^{2+} appears to be less potent than K^+/Ca^{2+} in the case of CCK-8 (see also Fig. 3).

Fig. 2 shows the time course of Ba^{2+}-evoked transmitter release. All transmitters showed an initial burst of release within the first seconds after addition of Ba^{2+} (within 15 s). This initial burst was more pronounced for the classical transmitters, glutamate and noradrenaline, than for the neuropeptide CCK-8. For glutamate the release in the first 15 s accounts for 80% of the total release. The Ba^{2+}-evoked release levelled off within 1 min after Ba^{2+} addition. No significant glutamate release was detected after 1 min in the continuous presence of Ba^{2+}. These release kinetics were similar to the K^+-evoked, Ca^{2+}-dependent release under similar conditions (Verhage et al., 1991c). For noradrenaline the initial release (15 s) accounted for 55% of all release. The Ba^{2+}-evoked release levelled off within 3 min after Ba^{2+} addition. No further noradrenaline release was detected after 1 min. Again, these release kinetics were similar to the K^+-evoked, Ca^{2+}-dependent release under similar conditions (Verhage et al., 1992a). Ba^{2+}-evoked CCK-8 release was also similar in kinetics as compared to K^+/Ca^{2+}-evoked release, with a characteristic, gradual build-up of release during the first minute after stimulation.

With respect to both the kinetics and extent of release, Ba^{2+} evoked transmitter release for all major transmitter classes closely resembled the K^+-evoked, Ca^{2+}-dependent release. In order to investigate whether both stimuli share the same cellular mechanisms and pools of transmitter, the additivity of both stimuli was investigated using sub-maximal doses of Ba^{2+} followed by maximal stimulation with K^+/Ca^{2+} and vice versa (Fig. 3). For glutamate, noradrenaline as well as CCK-8, 0.2 mM Ba^{2+} evoked release

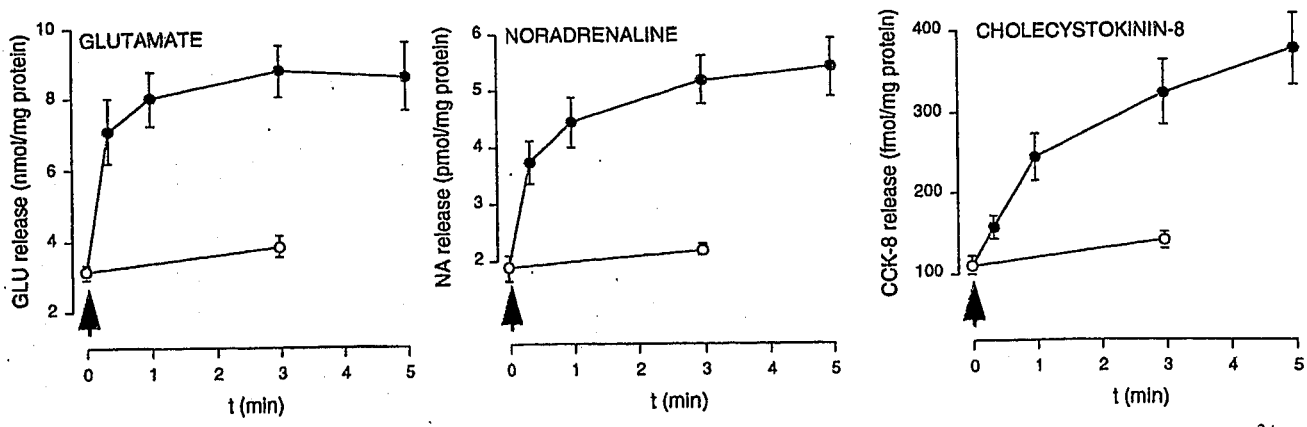

Fig. 2. Kinetics of Ba^{2+}-evoked release. Representatives of all major transmitter classes are shown. The arrow indicates addition of 1 mM Ba^{2+}. Data represent means ± S.E.M. of 4–5 independent preparations. The Ba^{2+}-evoked release (filled symbols) is significantly higher than basal efflux (in the absence of Ba^{2+}; open symbols) for all transmitters and all timepoints ($P < 0.01$) except in the case of the initial neuropeptide release. The initial release value, at the arrow, represents basal efflux and non-specific release during the 5 min pre-incubation period previous to Ba^{2+} addition (see Materials and methods).

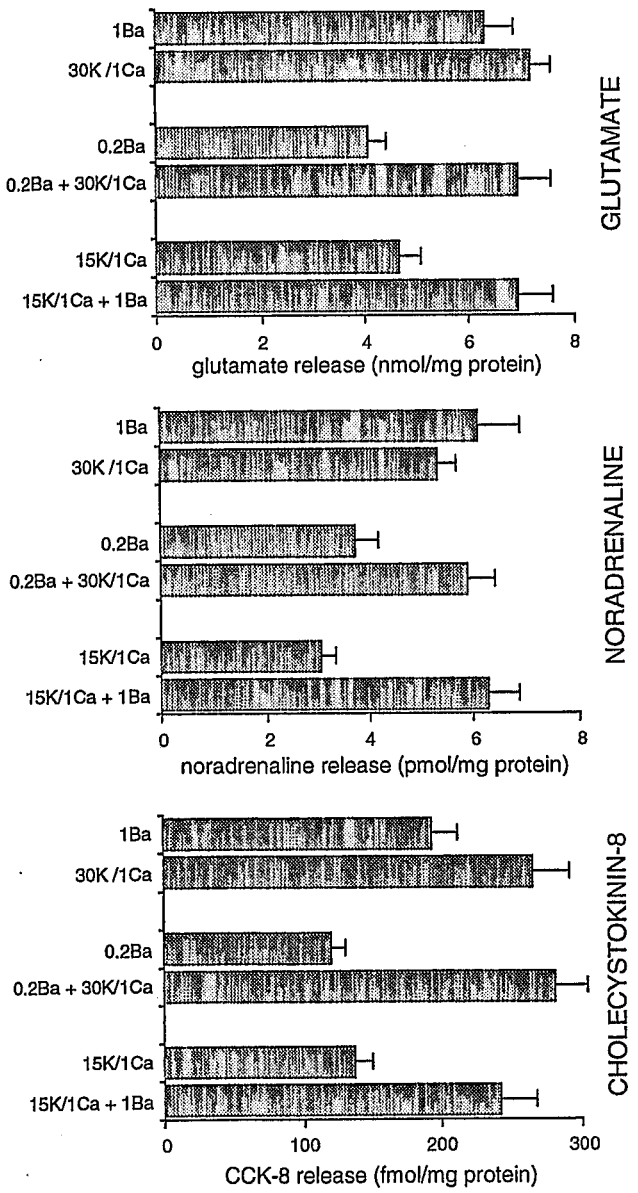

Fig. 3. The lack of additivity for Ba^{2+}- and K^+/Ca^{2+}-evoked transmitter release. Representatives of all major transmitter classes are shown. Bars represent means ± S.E.M. of 4–5 experiments. Release was estimated after 5 min preincubation followed by 3 min incubation with the indicated Ba^{2+} and/or K^+/Ca^{2+} concentration (see Materials and methods).

that was approximately half-maximal (Fig. 1), although Ba^{2+} tends to be less potent than Ca^{2+} in the case of CCK-8 (< 20%; significance: $P < 0.055$). Stimulation with 30 mM K^+ and 1 mM Ca^{2+}, in addition to Ba^{2+}, evoked additional release, but not more than 30 mM K^+/1 mM Ca^{2+} alone or the maximal release with 1 mM Ba^{2+} (upper two bars in each panel). Similarly, sub-maximal stimulation with 15 mM K^+/1 mM Ca^{2+} evoked a release that was approximately half-maximal. Stimulation with 1 mM Ba^{2+}, in addition to K^+/Ca^{2+}, evoked additional release, but again not more than 1 mM Ba^{2+} alone or 30 mM K^+/1 mM Ca^{2+}. The lack of additivity was found for all transmitter classes (Fig. 3).

3.2. The role of calmodulin-dependent processes in Ca^{2+}- and Ba^{2+}-dependent transmitter release

To investigate whether Ba^{2+} can mimic Ca^{2+}/calmodulin in stimulating protein phosphorylation ^{32}P incorporation into synaptosomal plasma membranes was measured. In the absence of Ca^{2+}, ^{32}P incorporation into synaptosomal proteins was generally very low (Fig. 4). Incubations in the presence of Ca^{2+} and calmodulin yielded large-scale phosphorylation over the total range of proteins, including the 50 kDa and 60 kDa bands, which correspond with the autophosphorylated Ca^{2+}/calmodulin kinase II subunits, and the 48 kDa band, the protein kinase C substrate B-50 (GAP-43). In contrast, incubation with 1 mM Ba^{2+} evoked in general little phosphorylation, also not in the 50 kDa and 60 kDa bands. Restricted increases in ^{32}P incorporation were found around 48 kDa (B-50/GAP-43) and around 90 kDa, a region where another major PKC substrate, MARCKS, migrates (Fig. 4). These increases were always relatively small as compared to the effects of Ca^{2+}/calmodulin. The Ca^{2+}/calmodulin-evoked phosphorylation was always more than 4-fold higher than the Ba^{2+}-evoked phosphorylation.

The role of calmodulin-dependent processes in transmitter release was further addressed by the use of the membrane-permeable calmodulin antagonist trifluoperizine. This agent was previously shown to inhibit transmitter release (see De Lorenzo, 1981) but may in high concentrations also interfere with the mitochondrial metabolism and hence inhibit transmitter release non-specifically by decreasing the high cellular ATP level, which is necessary for release (see Kauppinen et al., 1988). At the concentration of 10 μM, trifluoperizine did not induce a significant

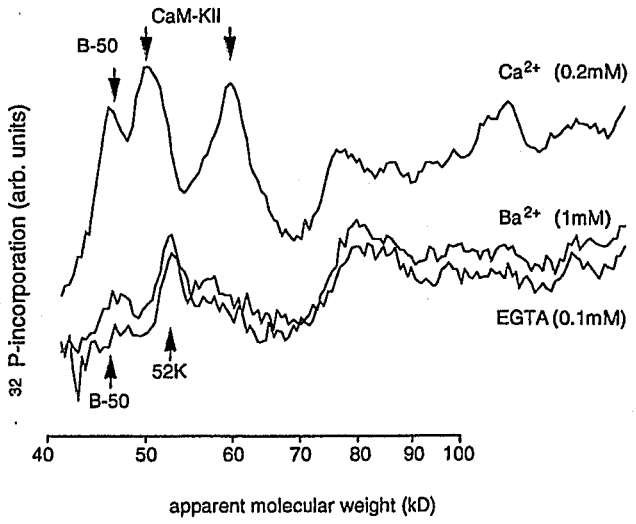

Fig. 4. Effect of Ba^{2+} and Ca^{2+} on protein phosphorylation in synaptosomal plasma membranes. Synaptosomal plasma membranes were incubated with the indicated ions or EGTA and proteins were separated by gel electrophoresis and quantified on a phosphoimager (see Materials and methods). Each trace represents the average of 3 incubations of a representative experiment.

decrease in cellular energy supplies as indicated by the ATP/ADP ratio. This ratio was between 4 and 5 both in the presence and absence of trifluoperizine ($n = 2$). This concentration of trifluoperizine nevertheless strongly inhibited K^+/Ca^{2+}-evoked transmitter release of all transmitter types (Fig. 5). The K^+-evoked release remaining in the presence of trifluoperizine largely reflects Ca^{2+}-independent release, caused by reversal of uptake carriers during persistent depolarization and non-specific leakage of transmitter during preincubation. This Ca^{2+}-independent release was measured in every assay, in order to calculate the net Ca^{2+}-dependent release (see Materials and methods) and amounted typically to 30–50% of the total release. This component was unaffected by trifluoperizine (data not shown). Hence, trifluoperizine abolished the Ca^{2+}-dependent exocytosis almost completely. In contrast, Ba^{2+}-evoked release was completely unaffected by 10 μM trifluoperizine (Fig. 5). The dose-response relation for

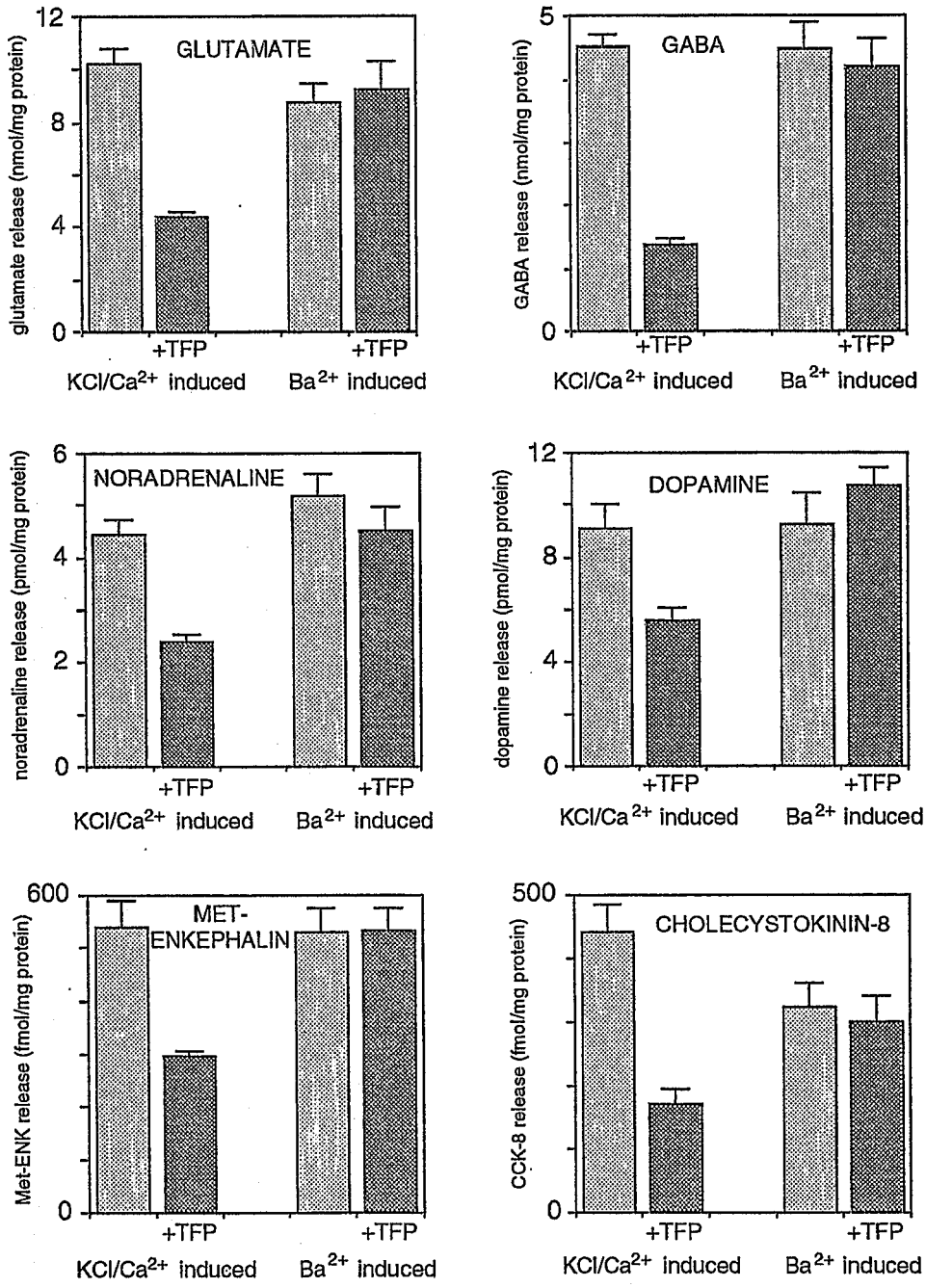

Fig. 5. The effects of trifluoperizine on Ba^{2+}- and K^+/Ca^{2+}-evoked transmitter release of all major transmitter classes. For all transmitters the K^+/Ca^{2+}-evoked release is significantly inhibited by trifluoperizine ($P < 0.01$), while the Ba^{2+}-evoked release is never significantly inhibited. Bars represent means ± S.E.M. of 3–4 experiments. Release was estimated after 5 min preincubation in the presence or absence of 10 μM trifluoperizine followed by 5 min incubation with either 1 mM Ba^{2+} or 30 mM K^+ in the presence of 1.5 mM Ca^{2+} (see Materials and methods).

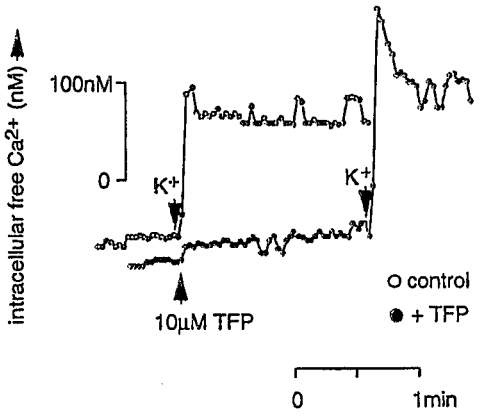

Fig. 6. Effects of trifluoperizine on the presynaptic Ca^{2+} regulation. Traces were shifted vertically in order to allow an optimal comparison of the transients. Additions were made as indicated by the arrows. $K^+ = 30$ mM KCl. Isolated nerve terminals were loaded with fura-2 and $[Ca^{2+}]_i$ was quantified as described in Materials and methods. Traces represent averages of 4 experiments derived from 2 independent preparations.

trifluoperizine showed that doses lower that 10 μM produced incomplete inhibition of transmitter release, whereas higher doses progressively affected cellular metabolism. Other calmodulin inhibitors (calmidazolium) could not be used without compromising synaptosomal viability. The inhibitory effect of trifluoperizine on K^+/Ca^{2+}-evoked release was not due to manipulation of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by trifluoperizine. Fig. 6 shows the $[Ca^{2+}]_i$ in fura-2-loaded isolated nerve terminals. Addition of trifluoperizine evoked a small increase in the resting $[Ca^{2+}]_i$, but did not alter the plateau phase of elevated $[Ca^{2+}]_i$ after K^+ depolarization in the presence of Ca^{2+} which is correlated to the release of the different transmitters (Verhage et al., 1991b; McMahon and Nicholls, 1991). The initial spike in $[Ca^{2+}]_i$ upon K^+ depolarization increased after preincubation with 10 μM trifluoperizine, emphasizing the role of calmodulin in buffering the initial Ca^{2+} spike upon depolarization. While the relevance of this initial spike in $[Ca^{2+}]_i$ for transmitter release is already doubtful (McMahon and Nicholls, 1991), the trifluoperizine-evoked *increase* in this spike cannot explain the trifluoperizine-evoked *decrease* in Ca^{2+}-dependent transmitter release.

Beside the calmodulin-dependent kinase II, calmodulin may also elicit its role in transmitter release through Ca^{2+}-dependent *de*phosphorylation (phosphatase 2B or calcineurin; see for a review Cohen, 1989). First we tested the effects of Ba^{2+} on calcineurin activity using purified components in a cell-free system. Fig. 7A shows the Ba^{2+}- and Ca^{2+}-evoked dephosphorylation of a prominent presynaptic calcineurin substrate, B-50 (GAP-43, Liu and Storm, 1989; Schrama et al., 1989) in the presence of calmodulin. Both Ba^{2+} and Ca^{2+} stimulate calcineurin activity. However, Ba^{2+} is less potent and requires a higher dose for maximal effectivity. At concentrations that evoke maximal release, around 1 mM Ba^{2+} (Fig. 1), the Ca^{2+}-evoked dephosphorylation is 2-fold higher than the Ba^{2+}-evoked dephosphorylation (Fig. 7A). Hence, the Ba^{2+}-sensitive calcineurin activity appears to be relatively small. Involvement of calcineurin activity in Ba^{2+}-evoked release was investigated in permeated synaptosomes, allowing introduction of antibodies. Ba^{2+}-evoked transmitter release was inhibited after blockade of calcineurin activity by introduction of anti-calcineurin IgGs (8 μg) into nerve terminals permeated with streptolysin-O (Hens et al., 1993). Ba^{2+}-evoked release of preloaded [^{3}H]noradrenaline from these terminals was inhibited by 20%. A slight inhibitory effect was also observed on basal release of [^{3}H]noradrenaline (Fig. 7B). Control experiments with introduction of total IgGs into permeated nerve terminals showed no effect on the release of [^{3}H]noradrenaline.

In addition, because the phosphatases type 1 and type 2A appear to be involved in stimulus-evoked and Ca^{2+}-dependent dephosphorylation in nerve terminals (Sim et al.,

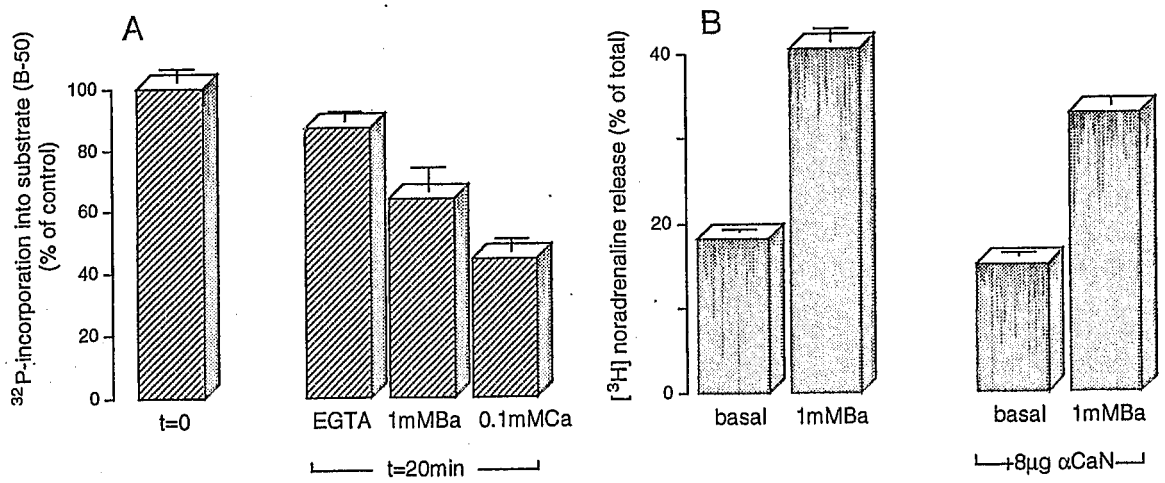

Fig. 7. Effects of Ba^{2+} on calcineurin activity and calcineurin-dependent transmitter release. (A) Ba^{2+} and Ca^{2+} induced dephosphorylation of the calcineurin substrate [^{32}P]B-50 (GAP-43) in cell-free experiments. (B) Inhibition of Ba^{2+}-evoked transmitter release after blockade of calcineurin activity by introduction of anti-calcineurin IgGs into permeated nerve terminals. Bars represent averages of 3 independent experiments ± S.E.M. in both panels.

1993), the effects of okadaic acid, an inhibitor of these phosphatases, was also tested (see Bialojan and Takai, 1988; Cohen et al., 1990). Okadaic acid potently inhibited both Ba^{2+}- and K^+/Ca^{2+}-evoked transmitter release (Fig. 8) with a half-maximal inhibition around 100 nM. No effect was found on the basal release of the different transmitters. The inhibition of GABA and [^{3}H]noradrenaline release was less pronounced than that of glutamate and CCK-8 release. Due to interference of okadaic acid with the assay conditions for endogenous catecholamines, the effects of okadaic acid on endogenous catecholamines could not be quantified. At 1 μM, the highest concentration used, okadaic acid did not decrease cellular ATP/ADP ratios and did not affect presynaptic $[Ca^{2+}]_i$ regulation (data not shown). The inhibition of evoked release by okadaic acid was found to be relatively labile, i.e., more sensitive to deterioration of the nerve terminal preparation than transmitter release itself. Whereas transmitter release is still significant in isolated nerve terminals 3–4 h after isolation, the okadaic acid-evoked inhibition is abolished. Instead, under these conditions okadaic acid tended to increase both basal and evoked release in a Ca^{2+}-independent manner. The potency of okadaic acid inhibition was found to correlate well with the initial burst of amino acid release, another feature of nerve terminals which is highly sensitive to deterioration (McMahon and Nicholls, personal communication). A similar correlation was found in the present experiments. Only preparations that also showed a potent initial amino acid release (> 1 nmol glutamate/mg protein/15 s; 2 of 7 preparations were excluded) were taken into account in the evaluation of the effects of okadaic acid.

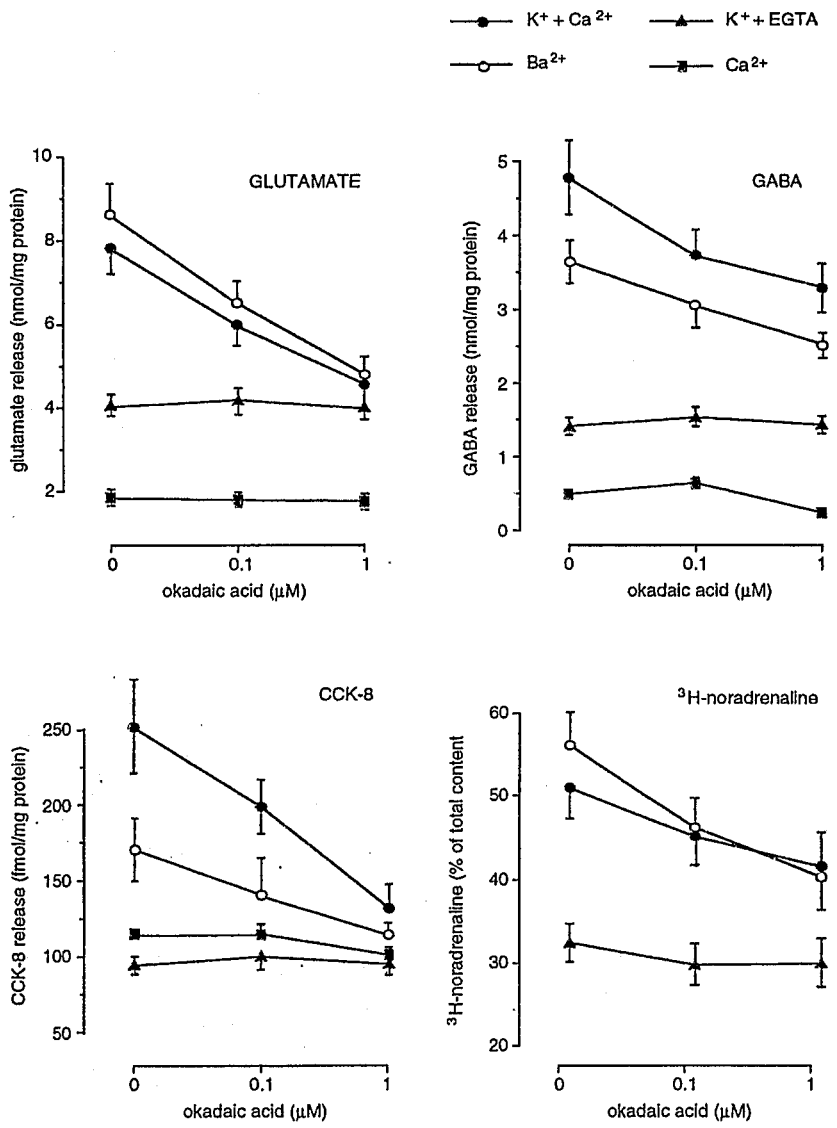

Fig. 8. Okadaic acid induced inhibition of endogenous amino acid and neuropeptide release and release of preloaded [^{3}H]noradrenaline. Isolated nerve terminals were preincubated with the indicated concentration of okadaic acid for 30 min. Release was estimated after 3 min further incubation with 1 mM Ca^{2+}, 1 mM Ba^{2+} or 30 mMK^+ with either 1 mM Ca^{2+} or 50 μM EGTA (see Materials and methods). Data represent averages of 3–6 independent preparations ± S.E.M.

4. Discussion

In the present study we have addressed three questions. Firstly, is Ba^{2+}-evoked release true exocytotic (vesicular) release? Secondly, is transmitter release independent of calmodulin activation and calmodulin-dependent protein phosphorylation? Thirdly, which cellular actions of Ba^{2+}, if not calmodulin activation and calmodulin-dependent protein phosphorylation, are responsible for its stimulatory effect on transmitter release. The present results confirm that Ba^{2+} ions, like Ca^{2+} ions, trigger transmitter release, as reported earlier (for instance Heldman et al., 1989; Tagliatela et al., 1989; Weiss et al., 1990; McMahon and Nicholls, 1993; Sihra et al., 1993). Ba^{2+} and Ca^{2+} were found to trigger release of all transmitters with similar kinetics and to a similar extent and to produce non-additive responses and thus appeared indistinguishable. However, in contrast to Ca^{2+}, Ba^{2+} evoked release which is insensitive to trifluoperizine and produced little protein phosphorylation. Both Ca^{2+}- and Ba^{2+}-evoked release were inhibited by okadaic acid. Furthermore, anti-calcineurin IgGs decreased the Ba^{2+}-evoked release.

4.1. Is Ba^{2+}-evoked release true exocytotic (vesicular) release?

Ba^{2+} ions exploit their permeability in Ca^{2+} channels and probably other, non-specific pathways to enter the terminal (Sihra et al., 1993). Ba^{2+} does not occur in high concentrations in the brain, has no known function in signal transduction in vivo, does not bind to many soluble Ca^{2+}-buffering components and is sequestered within and/or extruded from the nerve terminal to a very limited extent (Chao et al., 1984; Rasgado-Flores et al., 1987; Mironov and Juri, 1990). This lack of cellular components regulating the intracellular Ba^{2+} concentration and the limited size of a terminal (1 μm diameter) will allow Ba^{2+} to accumulate and equilibrate rapidly within the terminal and evoke release, also in polarized nerve terminals (see also Tagliatela et al., 1989; Sihra et al., 1993). This equilibration of Ba^{2+} may be different from the subcellular Ca^{2+} distribution, which may show regional variations within the terminal (see for a review Verhage et al., 1995). It has been shown that the entrance of Ba^{2+} into the terminal, potentially in concert with its inhibitory action on K^+ permeabilities, depolarizes the nerve terminal and hereby facilitates its own entrance through voltage-gated Ca^{2+} channels (McMahon and Nicholls, 1993; Sihra et al., 1993). This depolarization may also result in tetrodotoxin-sensitive instability of the membrane potential (spiking) in isolated nerve terminals (McMahon and Nicholls, 1993). In order not to complicate the interpretation of the effects of Ba^{2+} on release, rather than on the membrane potential, tetrodotoxin was included in all experiments in the present study. Furthermore, as we show here, Ba^{2+} also evokes transmitter release in permeated synaptosomes (Fig. 7, see also Kish and Ueda, 1991). Hence, the actions of Ba^{2+} on transmitter release cannot be explained by direct or indirect effects of Ba^{2+} on presynaptic membrane potential and/or ionic balance or indirect effects of Ba^{2+} by exchanging with Ca^{2+} accumulated in internal compartments. Instead, the lack of additivity in the effects of Ca^{2+} and Ba^{2+} as well as the comparable release profiles upon stimulation suggest that Ba^{2+}, similar to Ca^{2+}, directly stimulates transmitter release. The lack of additivity indicates that at least shared steps exist in the way both ions produce release and probably share the same pool of transmitter. This holds true for all the transmitter types, indicating a conserved aspect among the release mechanisms for different transmitters from different vesicles. Taken together, our data support the conclusion that Ba^{2+} evokes true exocytotic (vesicular) release.

Interestingly, whereas Ca^{2+} requirements may differ for the release of different transmitters in a similar preparation (see Verhage et al., 1991b, 1995), the effective concentration of Ba^{2+} was rather similar for different transmitters, whether they are released from small synaptic vesicles at the active zone or from large dense cored vesicles at more ectopic sites within the terminals. As discussed above, Ba^{2+} may, in contrast to Ca^{2+}, equilibrate rapidly within the terminal. This may explain why Ba^{2+} evokes release with similar effectivity for both types of vesicle. Consequently, the apparently different Ca^{2+} sensitivity of the release mechanisms of different transmitters (Verhage et al., 1991b) may be explained by different local Ca^{2+}-buffering capacities and different coupling between the sites of Ca^{2+} entry and release rather than by intrinsic differences in the affinity for divalent cations.

4.2. Is transmitter release indeed independent of calmodulin activation and calmodulin-dependent protein phosphorylation?

The inability of Ba^{2+} to activate calmodulin has been considered as an argument to question the true exocytotic nature of Ba^{2+}-evoked release (see for discussion Robinson, 1992; McMahon and Nicholls, 1993; Verhage et al., 1995). However, the present results using calmodulin and phosphatase inhibition show that Ba^{2+} may act downstream of calmodulin activation: Ca^{2+}- and not Ba^{2+}-evoked release is blocked by a calmodulin antagonist, while both Ca^{2+}- and Ba^{2+}-evoked release are blocked by inhibition of phosphatase activity. Again, this mechanism appears to be shared by all major transmitter types. The inhibition of release by trifluoperizine confirms earlier suggestions implicating calmodulin in the release process (see De Lorenzo, 1981). Although trivial effects of the calmodulin inhibitor trifluoperizine on presynaptic energy metabolism or $[Ca^{2+}]_i$ regulation were excluded in the present study, the specificity and effectivity of blocking calmodulin actions may be questioned. Pharmacological tools to manipulate calmodulin are still limiting. However,

the differential effect of trifluoperizine, effectively blocking Ca^{2+}-evoked release, while ineffective in blocking Ba^{2+}-evoked release, excludes non-specific effects of the drug on presynaptic function. Our results are at this point at variance with Sitges and Talamo (1993) describing profound effects of trifluoperizine on $[Ca^{2+}]_i$ regulation. This difference may arise from different preparations used, since the capacity to regulate the steady state $[Ca^{2+}]_i$ is rather different among different preparations (Verhage et al., 1988).

Although calmodulin itself may be involved in triggering transmitter release, the relevance of calmodulin-dependent phosphorylation in the same process appears to be rather limited, since Ba^{2+} is able to support release of all transmitters while it is largely ineffective in stimulating kinase activity. Especially major presynaptic substrates for calmodulin-dependent phosphorylation, such as the calmodulin-kinase II itself (Fig. 4) and the synapsins (see Sihra et al., 1993) appear not to incorporate ^{32}P after application of Ba^{2+}. In conclusion, Ba^{2+} may replace the Ca^{2+}-calmodulin complex rather than Ca^{2+} alone in triggering transmitter release. The relevance of protein phosphorylation for the events prior to transmitter release may be questioned. Several recent reports, using a variety of approaches, indicate that the Ca^{2+}-dependent phosphorylation of presynaptic proteins may indeed be of limited importance for initiating transmitter release (see Hens et al., 1993; McMahon and Nicholls, 1993; Rosahl et al., 1993).

4.3. Which cellular actions of Ba^{2+} are responsible for its stimulatory effect on transmitter release?

Rather than stimulating presynaptic protein phosphorylation, Ba^{2+} should have other cellular effects leading to transmitter release. One potential effector is the synaptic vesicle protein synaptotagmin. Synaptotagmin has been shown to undergo Ca^{2+}-dependent changes (Brose et al., 1992) and to be essential for presynaptic function (see Popov and Poo, 1993). The Ca^{2+}-binding domain of this protein binds to phospholipids upon increases in Ca^{2+} and also in Ba^{2+} (Davletov and Südhof, 1993), and may therefore be a common target of both Ca^{2+} and Ba^{2+} to evoke release. In addition, protein phosphatases appear to play an essential role in the steps leading to both Ca^{2+}- and Ba^{2+}-dependent transmitter release. This role may be indirect and may also involve recruitment of transmitters and synaptic vesicles for release, i.e., recycling and docking of synaptic vesicles. The most profound effect is found with okadaic acid, which requires long preincubations in order to block phosphatases 1 and 2A in intact systems. Furthermore, the present study has focused on the total Ca^{2+}-dependent release during one period of permanent depolarization. Eventually, protein phosphorylation should be required in order to produce substrates for the phosphatases implicated here in transmitter release. Indeed, preliminary results indicate that Ba^{2+}-evoked neuropeptide release displays a profound run-down in experiments with multiple periods of stimulation in a perfusion set-up, while successive K^+/Ca^{2+}-evoked releases exhibit comparable amplitudes. This may indicate that the recycling of transmitter vesicles is only supported by Ca^{2+} and Ca^{2+}-dependent phosphorylation, and not by Ba^{2+}.

The relevant targets for this dephosphorylation remain obscure. Most of the major presynaptic proteins, as can be identified in total protein electrophoresis of synaptic plasma membranes or synaptosomal preparations, primarily undergo large-scale phosphorylation upon stimulation and $[Ca^{2+}]_i$ elevation (Fig. 4, see also Wang et al., 1988; Robinson, 1992). One protein that shows initial dephosphorylation, p96 or dynamin-I, is implicated in endocytosis rather than exocytosis (see Robinson et al., 1993). The present results support earlier findings suggesting that the intrinsically Ca^{2+}-independent phosphatases 1 and 2A are involved in the Ca^{2+}-dependent dephosphorylation upon stimulation (Sim et al., 1991, 1993). In addition, calcineurin (phosphatase 2B, see Cohen, 1989) also appears to be involved in both Ba^{2+}- and Ca^{2+}-evoked transmitter release, although Ba^{2+} is less potent in activating calcineurin and Ba^{2+}-evoked release cannot be completely blocked by introduction of α-calcineurin IgGs in permeated synaptosomes. However, the inhibitory effect of the IgGs may be underestimated because not all nerve terminals can be permeated without compromising the viability of the preparation and anti-calcineurin-resistant release after Ba^{2+} application may in fact arise from this population of non-permeated terminals. Furthermore, antibodies may not produce complete interference with the function of its antigen and the epitope may not be optimally accessible. For these reasons, we cannot exclude that the role of calcineurin in Ba^{2+}-evoked release is more pronounced in vivo. This would be in line with earlier reports (for instance: Lisman, 1989; Sim et al., 1993) suggesting that Ca^{2+}-dependent dephosphorylation of endogenous inhibitors regulates the activity of intrinsically Ca^{2+}-independent phosphatases involved in stimulus-evoked events. Hence, the present results stress the importance of Ca^{2+}-dependent or independent *de*phosphorylation, rather than phosphorylation in presynaptic transmitter release. This observation is in line with reports from exocytosis in several non-neuronal systems such as chromaffin cells (Cote et al., 1986), islet cells (Jones et al., 1988) and *Paramecium* (Plattner, 1989).

Acknowledgements

The authors thank Mrs. Ank Frankhuijzen-Sierevogel and Elly Besselsen for their skilful technical assistance. Drs. K. Kissmehl and H. Plattner are acknowledged for their kind supply of anti-calcineurin IgGs and Dr. G.J. Dockray for CCK-8 antiserum. J.J.H.H. was supported by

grant 903-53-091 of the Netherlands Organization of Scientific Research (MW-NWO).

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