British Journal of Pharmacology (2000) 129, 1780–1786
www.nature.com/bjp

Release of dopamine from human neocortex nerve terminals evoked by different stimuli involving extra- and intraterminal calcium

[1]Giambattista Bonanno, [1]Roberta Sala, [1]Laura Cancedda, [2]Paolo Cavazzani, [3]Massimo Cossu & *,[1]Maurizio Raiteri

[1]Dipartimento di Medicina Sperimentale, Sezione di Farmacologia e Tossicologia, Università di Genova, Viale Cembrano 4, 16148 Genova, Italy; [2]Divisione di Neurochirurgia, Ospedali Galliera, Via A. Volta 8, 16128 Genova, Italy and [3]Clinica Neurochirurgica, Università di Genova, Ospedale S. Martino, Largo Rosanna Benzi 10, 16132 Genova, Italy

1 The release of [^{3}H]-dopamine ([^{3}H]-DA) from human neocortex nerve terminals was studied in synaptosomes prepared from brain specimens removed in neurosurgery and exposed during superfusion to different releasing stimuli.

2 Treatment with 15 mM KCl, 100 μM 4-aminopyridine, 1 μM ionomycin or 30 mM caffeine elicited almost identical overflows of tritium. Removal of external Ca^{2+} ions abolished the overflow evoked by K^+ or ionomycin and largely prevented that caused by 4-aminopyridine; the overflow evoked by caffeine was completely independent of external Ca^{2+}.

3 Exposure of synaptosomes to 25 μM of the broad spectrum calcium channel blocker $CdCl_2$ strongly inhibited the 4-aminopyridine-induced tritium overflow while that evoked by ionomycin remained unaffected.

4 The Ca^{2+} chelator, 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid (BAPTA), reduced significantly the K^+- and the caffeine-induced tritium overflow. The effect of caffeine was attenuated by exposure to the ryanodine receptor blocker dantrolene or when the membrane-impermeant inositol trisphosphate receptor antagonist, heparin, was entrapped into synaptosomes; the combined treatment with dantrolene and heparin abolished the release elicited by caffeine.

5 Tetanus toxin, entrapped into human neocortex synaptosomes to avoid prolonged incubation, inhibited in a concentration-dependent manner the K^+- or the 4-aminopyridine-evoked tritium overflow; in contrast, the release stimulated by ionomycin and by caffeine were both totally insensitive to the same concentrations of tetanus toxin. Western blot analysis showed about 50% reduction of the content of the vesicular protein, synaptobrevin, in synaptosomes poisoned with tetanus toxin.

6 In conclusion, the release of dopamine from human neocortex nerve terminals can be triggered by Ca^{2+} ions originating from various sources. It seems that stimuli not leading to activation of voltage-sensitive Ca^{2+} channels elicit Ca^{2+}-dependent, probably exocytotic, release that is insensitive to tetanus toxin.

British Journal of Pharmacology (2000) **129,** 1780–1786

Keywords: Human neocortex; dopamine release; tetanus toxin; calcium pools; exocytosis

Abbreviations: CICR, Ca^{2+}-induced Ca^{2+} release; DA, dopamine; $InsP_3Rs$, inositol trisphosphate receptors; RYRs, ryanodine receptors; SDS, sodium dodecyl sulphate; TeTx, tetanus toxin; VSCC, voltage-sensitive calcium channel

Introduction

Influx of Ca^{2+} ions into nerve terminals through voltage-sensitive calcium channels (VSCCs) is the classical mechanism of transmitter release. When VSCCs open during depolarization, a microdomain of Ca^{2+} ions forms around the docked vesicles resulting in successful vesicle fusion and transmitter exocytosis. There is increasing evidence that exocytosis can also occur independently of VSCC activation, following local release of Ca^{2+} from internal stores (Blöchl & Thoenen, 1995; Peng, 1996; Smith & Cunnane, 1996; Tse *et al*., 1997; see Berridge, 1998). Internal Ca^{2+} can be released into the cytoplasm by various interconnected mechanisms, including release from inositol trisphosphate receptors ($InsP_3Rs$), from ryanodine receptors (RYRs) and from mitochondria (see, for a review, Berridge, 1998). Mechanisms leading to Ca^{2+} mobilization from internal stores can be activated by endogenous stimuli, such as agonist binding at metabotropic receptors, but also by pathological conditions and treatments with drugs. The possibility that vesicular exocytosis of neurotransmitters is triggered by Ca^{2+} originating from different sources and the involvement in exocytosis of an ever growing number of presynaptic proteins have increased tremendously the complexity of this process. We are investigating on the hypothesis that exocytosis may differ in some aspects when different neuronal families are exposed to the same releasing stimulus and/or when the same neuronal family is exposed to different releasing stimuli.

The present work was performed with synaptosomes prepared from fresh human neocortical samples, labelled with [^{3}H]-dopamine ([^{3}H]-DA) and exposed in superfusion to multiple releasing stimuli able to cause quantitatively comparable tritium overflows. The stimuli applied are: high-K^+ and 4-aminopyridine, expected to elicit depolarization gating Ca^{2+} influx through VSCCs; ionomycin, a Ca^{2+}-selective ionophore able to mediate direct Ca^{2+} influx into the terminals, thereby bypassing VSCCs and the need for membrane depolarization; caffeine, a drug that mobilizes Ca^{2+} in neurons by acting at RYRs on the endoplasmic reticulum (Thayer *et al*., 1988; McPherson *et al*., 1991; Friel & Tsien, 1992). We studied the relations between dopamine release and pools of Ca^{2+} involved; we also tested the sensitivity to tetanus

*Author for correspondence; E-mail: raiteri@pharmatox.unige.it

toxin (TeTx) of the DA overflow provoked by the different releasing stimuli.

Methods

Human samples

Human cerebral cortex specimens were obtained from three female and ten male patients (aged 20–60 years) undergoing neurosurgery to reach deeply located tumours (four cases; specimens were part of the frontal lobe) or suffering untreatable temporal lobe epilepsy (nine cases). The tissues were obtained in different days and processed on the day of the surgery. Since we did not observe significant differences between results obtained from the two groups of patients, all data have been pooled.

Synaptosomes preparation

Immediately after removal, tissues were placed in a physiological salt solution (see below) kept at 0–4°C and synaptosomal preparations were obtained within 60–90 min. Tissue homogenization was performed in 2 ml 0.32 M sucrose, buffered at pH 7.4 with phosphate, in the presence or in the absence of TeTx (100–500 nM), 1,2-bis-(2-aminophenoxy) ethane-N,N,N′,N′ tetraacetic acid (BAPTA; 1 mM) or heparin (40 μM), using a glass-teflon tissue grinder (clearance 0.25 mm, 12 up-down strokes in about 1 min, 900 r.p.m.). The homogenate was first centrifuged at 1000 × *g* for 5 min; synaptosomes were isolated from the supernatant by centrifugation at 12 000 × *g* for 20 min. All the preceding procedures were performed at 0–4°C. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (in mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 22 and glucose 10 (aeration with 95% O_2 and 5% CO_2), pH 7.2–7.4 and incubated for 15 min at 37°C with 0.1 μM [^{3}H]-dopamine. Labelling was performed in the presence of 0.1 μM 6-nitroquipazine and desmethylimipramine, to avoid false labelling of serotonergic or noradrenergic terminals, respectively. Synaptosomal protein was measured according to Bradford (1976).

Release experiments

Aliquots of the synaptosomal suspensions (about 0.2 mg of synaptosomal protein) were distributed on microporous filters placed at the bottom of 20 parallel superfusion chambers maintained at 37°C (Raiteri *et al*., 1974). Superfusion was then started with standard medium at a rate of 0.5 ml min^{-1} and continued for a total of 50 min. After 34 min to equilibrate the system, fractions were collected according to the following scheme: two 4 min fractions (t = 34–38 and t = 46–50 min, respectively; basal release) before and after one 8 min fraction (t = 38–46 min; evoked release). At t = 38, synaptosomes were exposed to a 90 s pulse of 15 mM KCl, 100 μM 4-aminopyridine, 1 μM ionomycin or 30 mM caffeine. $CdCl_2$ or dantrolene was added to the superfusion medium at t = 30 min. Calcium-deprived medium was introduced at t = 20 min. At the end of the experiment, fractions collected and superfused filters were counted for their tritium content.

Calculations

The amount of radioactivity released in each fraction was expressed as a per cent of the total tritium content at the start of the respective collection period. Stimulus-induced overflow was estimated by subtracting the percentage of tritium content in the two 4 min fractions representing the basal release from the release evoked in the 8 min fraction collected during and after the 90 s stimulation period. Drug effects were evaluated as the ratio of the stimulation-evoked overflow calculated in the presence of drugs versus that calculated under control conditions. Appropriate controls were always run in parallel. Two-tailed Student's *t*-test was used for comparison of two mean values.

Immunoblotting

Synaptosomal proteins from control and 500 nM TeTx-treated synaptosomes were subjected to polyacrylamide gel electrophoresis followed by Western blotting to measure syntaxin and synaptobrevin content. Electrophoresis on sodium dodecyl sulphate (SDS)-12% (w v^{-1}) polyacrylamide minigel was performed according to the method of Laemmli (1970), loading 20 μg protein per lane. Transfer to nitrocellulose membrane was performed electrophoretically at 30 V overnight. The membrane was then treated with bovine serum albumin to block non specific binding, cut near the 30 kDa molecular marker and incubated for 3 h with anti-syntaxin monoclonal antibody (MAB336; 1 : 20000; upper part) or with anti-synaptobrevin monoclonal antibody (MAB333; 1 : 1000; lower part). The membranes were washed and incubated (45 min) with horseradish peroxidase conjugated (sheep) anti-mouse Ig whole antibody diluted 1 : 2000. After the latter incubation, membranes were evenly coated using the ECL Western blotting detection system from 1 min. The membranes were immediately exposed to Amersham autoradiography film at room temperature for various periods (15–60 s). Blots were scanned using a computerized densitometer.

Drugs

7,8[^{3}H]-Dopamine (sp. act. 43 Ci mmol^{-1}), horseradish peroxidase-conjugated anti-mouse Ig whole antibody prestain molecular mass marker and ECL detection system were purchased from Amersham Radiochemical Centre (Buckinghamshire, U.K.); 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid (BAPTA) from Fluka Biochemika (Milan, Italy). Caffeine, 4-aminopyridine and dantrolene, sodium salt, were obtained from Sigma (St. Louis, MO, U.S.A.). Ionomycin free acid and heparin sodium salt were obtained from Calbiochem (La Jolla, CA, U.S.A.). Mouse anti-synaptobrevin monoclonal antibody (MAB 333) and mouse anti-syntaxin monoclonal antibody (MAB 336) were obtained from Chemicon (Temecula, CA, U.S.A.). Tetanus toxin was kindly donated by Dr Ulrich Weller (Johannes Gütenberg Universität, Mainz, Germany). The following drugs were generous gifts by the companies indicated: 6-nitroquipazine maleate (Duphar, Weesp, The Netherlands) and desmethilimipramine (Novartis, Basel, Switzerland).

Results

Release of [^{3}H]-dopamine from human neocortex synaptosomes exposed to different stimuli

Human neocortex synaptosomes were prelabelled with [^{3}H]-DA and the tritium efflux was measured in superfusion. When a 90 s pulse of 15 mM KCl was applied, this relatively mild depolarizing condition caused significant tritium overflow

(total minus basal release). This overflow was completely dependent on external Ca^{2+} (Figure 1). The Figure also shows that a 90 s pulse of 100 μM 4-aminopyridine produced the same overflow as 15 mM K^+. Also in this case removal of external Ca^{2+} largely inhibited the overflow of tritium. The effect of 4-aminopyridine was strongly prevented by Cd^{2+}, a broad spectrum Ca^{2+} channel blocker (Table 1). These results suggest that membrane depolarization by KCl or 4-aminopyridine gates Ca^{2+} influx through VSCCs and that Ca^{2+} triggers in turn Ca^{2+}-dependent dopamine exocytosis. Ionomycin, a Ca^{2+}-selective ionophore able to mediate direct Ca^{2+} influx into nerve terminals independently of depolarization and VSCC activation, was then applied. Similar to K^+ or 4-aminopyridine, ionomycin (1 μM; 90 s) caused tritium overflow which was quantitatively identical to the overflow provoked by K^+ or 4-aminopyridine and only occurred in the presence of Ca^{2+} in the superfusion medium (Figure 1). As reported in Table 1, the overflow produced by 1 μM ionomycin was insensitive to Cd^{2+}, in line with the reported inability of ionomycin to depolarize synaptosomes (and activate VSCCs) at the low concentration used here (Verhage *et al*., 1991). Human neocortex synaptosomes were also exposed to a 90 s pulse of caffeine (30 mM) which elicited a tritium overflow quantitatively similar to that caused by the other stimuli applied, but completely independent of external Ca^{2+}.

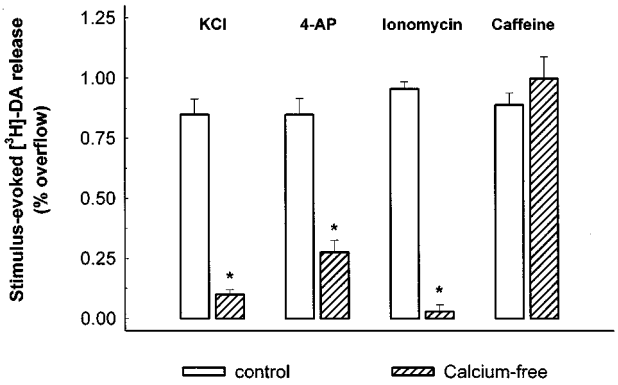

Figure 1 Overflows of [^{3}H]-dopamine ([^{3}H]-DA) induced by 15 mM KCl, 100 μM 4-aminopyridine (4-AP), 1 μM ionomycin or 30 mM caffeine from human brain cortex synaptosomes in superfusion and their Ca^{2+}-dependency. A 90 s period of stimulation with KCl, 4-AP, ionomycin or caffeine was applied after 38 min of superfusion. When appropriate, Ca^{2+} was omitted 18 min before the different release stimuli. The overflows were estimated by subtracting the percentage tritium content in the two 4 min fractions, representing the basal release, from the release evoked in the 8 min fraction collected during and after the stimulation period. The basal release amounted to 3.4 ± 0.24% of the total synaptosomal content ($n=4$). Each bar represents the mean $\pm$ s.e.mean of four experiments run in triplicate. $^*P<0.001$ when compared to respective control value (two-tailed Student's *t*-test).

Table 1 Effect of $CdCl_2$ on the overflow of [^{3}H]-dopamine induced by 4-aminopyridine or ionomycin from human brain cortex synaptosomes

	%overflow of [^{3}H]-dopamine	
	Control	*25 μM $CdCl_2$*
4-Aminopyridine (100 μM)	0.70 ± 0.07 (4)	0.15 ± 0.02 (4)*
Ionomycin (1 μM)	1.02 ± 0.04 (3)	1.10 ± 0.02 (3)

A 90 s period of stimulation was applied after 38 min of superfusion $CdCl_2$ was introduced 8 min before. Mean $\pm$ s.e.mean of *n* (in parentheses) experiments run in triplicate are reported. $^*P<0.001$ when compared with respective control value (two-tailed Students *t*-test)

Role of calcium in the caffeine-induced [^{3}H]-dopamine release

Caffeine is expected to release Ca^{2+} from internal stores, thus elevating cytoplasmic Ca^{2+} concentration. However, to our knowledge, caffeine has not been reported to release dopamine from animal brain tissues. It appeared of interest, therefore, to characterize in more detail the mechanism of action of caffeine as a dopamine releaser in dopaminergic terminals of the human meso-cortical pathway.

As shown in Figure 2, dantrolene (1 μM), a drug known to inhibit Ca^{2+} release from intracellular stores, probably the ryanodine-sensitive ones (Frandsen & Schousboe, 1992; Simpson *et al*., 1995; Parness & Palnitkar, 1995), attenuated the caffeine-evoked release of tritium. Heparin is a competitive antagonist of $InsP_3$ receptors (Finch *et al*., 1991; Taylor & Richardson, 1991). Since heparin is membrane-impermeant, the compound was incorporated into synaptosomes by homogenizing human neocortical tissue in buffered sucrose containing 40 μM heparin, according to a technique originally devised to entrap small molecules into subsequently isolated synaptosomes (Åkerman & Heinonen, 1983), but recently shown to permit incorporation also of molecules of large size (Raiteri *et al*., 2000). Figure 2 shows that the caffeine-induced release of tritium from human synaptosomes was attenuated by heparin entrapped at the estimated concentration of $\leqslant 2$ μM ($\leqslant 5$% of the concentration originally present in the homogenization medium based on experiments with [^{3}H]-sucrose; Åkerman & Heinonen, 1983). The combined action of dantrolene and heparin almost abolished the caffeine-evoked release of tritium (Figure 2). The Ca^{2+} chelator, BAPTA, entrapped in its impermeant acid form (100 μM in the homogenization medium; internal concentration ~ 5 μM), in order to avoid the possible inhibition of K^+ currents reported to occur when using the permeant form BAPTA-AM (Watkins & Mathie, 1996), largely inhibited the caffeine-evoked tritium overflow, although less effectively than the K^+ (15 mM)-evoked overflow. Treatment with dantrolene plus entrapped heparin and BAPTA completely prevented the release of tritium elicited by caffeine (Figure 2).

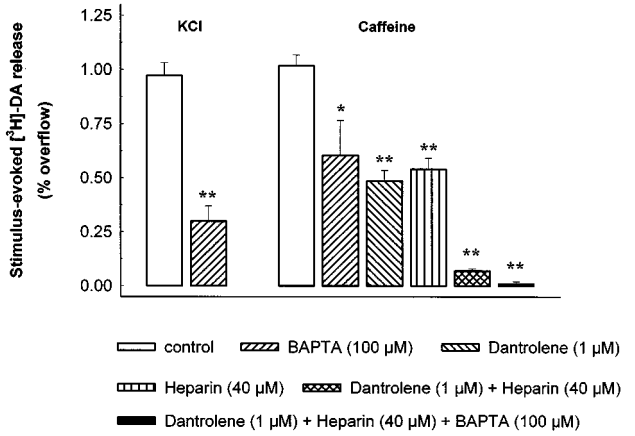

Figure 2 Effect of BAPTA, dantrolene and heparin on the overflow of [^{3}H]-dopamine induced by 15 mM KCl or 30 mM caffeine from human brain cortex synaptosomes. Synaptosomes were prepared by adding BAPTA and/or heparin during homogenization of the tissue to entrap these impermeant reagents. Dantrolene was added during superfusion. A 90 s period of stimulation with KCl or caffeine was applied after 38 min of superfusion. Dantrolene was introduced 8 min before the different release stimuli. Each bar represents the mean $\pm$ s.e.mean of 3–4 experiments run in triplicate. $^*P<0.005$; $^{**}P<0.001$, when compared to respective control value (two-tailed Student's *t*-test).

Effect of tetanus toxin on the release of [^{3}H]-dopamine induced by different stimuli

The effect of TeTx, a clostridial toxin able to cleave VAMP/synaptobrevin (Link *et al*., 1992; Schiavo *et al*., 1992), on the release of DA evoked from human neocortex synaptosomes by high-K^+, 4-aminopyridine, ionomycin and caffeine was then analysed. It has been observed that human brain synaptosomes are less viable than synaptosomes quickly prepared from animal brain. In preliminary experiments, human synaptosomes obtained within 60–90 min from neurosurgery and incubated 90 min with TeTx showed inconsistent release of previously taken up [^{3}H]-DA. Since we recently demonstrated that TeTx can be successfully entrapped into rat synaptosomes, where it can inhibit K^+-evoked GABA and glutamate release (Raiteri *et al*., 2000), we exploited the entrapping technique to bypass the 90 min incubation of human synaptosomes with TeTx. Figure 3 shows that the overflow of [^{3}H]-DA provoked by 15 mM KCl or by 100 μM 4-aminopyridine was concentration-dependently depressed by TeTx (100 or 500 nM in the homogenization medium). In contrast, neither the tritium release provoked by 1 μM ionomycin nor that provoked by 30 mM caffeine could be significantly reduced by entrapped TeTx.

Cleavage of synaptobrevin by tetanus toxin in human neocortex synaptosomes

Cleavage of human VAMP/synaptobrevin by TeTx was investigated by SDS–PAGE of synaptosomal proteins followed by Western blotting with an anti-synaptobrevin monoclonal antibody. As a control, in the same blots the synaptosomal content of syntaxin, a protein not cleaved by TeTx (Link *et al*., 1992; Williamson *et al*., 1996), was analysed; synaptobrevin levels were normalized to the corresponding syntaxin band. The results of Figure 4 show that the synaptobrevin content was reduced by about 50% in human synaptosomes prepared by homogenization in the presence of 500 nM TeTx, similar to what had been found in *rat* synaptosomes staffed with the same maximally effective concentration of TeTx (Fassio *et al*., 1999).

Discussion

Many studies, essentially performed with animal brain tissues, have shown that high-K^+ and 4-aminopyridine are depolarizing agents which, by different mechanisms, lead to activation of VSCCs in nerve terminals, entry of Ca^{2+} ions and release of neurotransmitters by conventional exocytosis. This picture seems to hold also in the case of DA release from the terminals of human mesocortical neurons. Indeed, the K^+-evoked

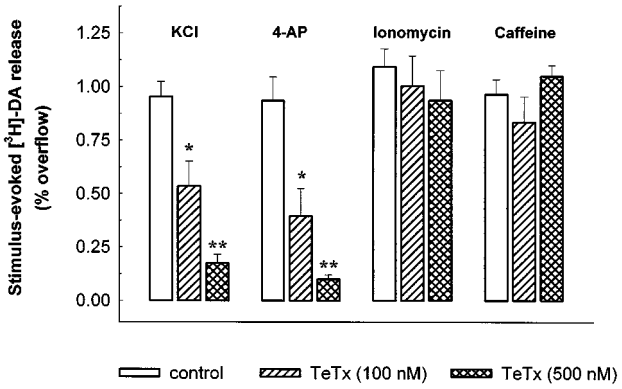

Figure 3 Effect of tetanus toxin (TeTx) on the overflow of [^{3}H]-DA induced 15 mM KCl, 100 μM 4-aminopyridine (4-AP), 1 μM ionomycin or 30 mM caffeine from human brain cortex synaptosomes. Synaptosomes were prepared with TeTx present during homogenization of the tissue. A 90 s period of stimulation with KCl, 4-AP, ionomycin or caffeine was applied after 38 min of superfusion. Each point represents the mean±s.e.mean of 3–4 experiments run in triplicate. $*P<0.005$, $**P<0.001$ when compared to respective control value (two-tailed Student's *t*-test).

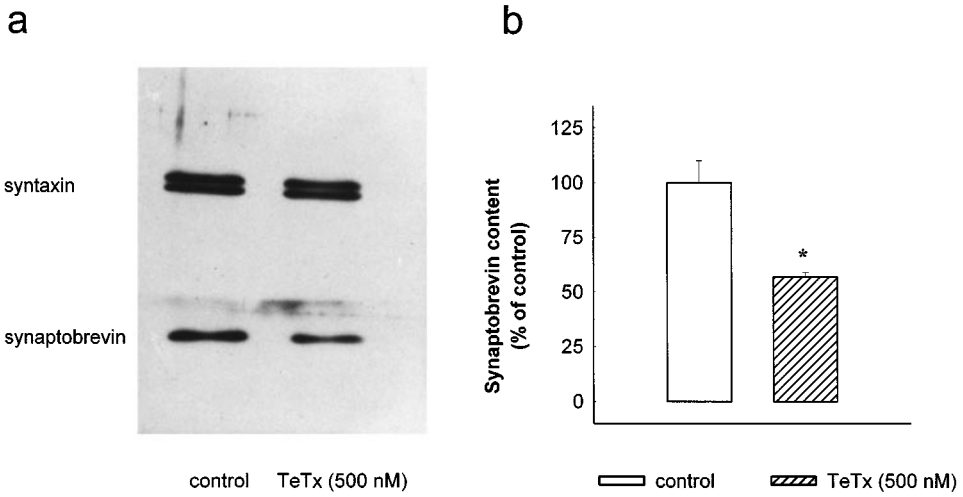

Figure 4 Cleavage of synaptobrevin in human brain cortex synaptosomes by entrapped tetanus toxin (TeTx). (a) representative blot from control synaptosomes and synaptosomes prepared by homogenization in the presence of TeTx. Synaptosomal protein (20 μg per lane) were subjected to SDS–PAGE (12% gel) followed by Western blotting using a mouse anti-synaptobrevin monoclonal antibody (MAB 333) or a mouse anti-syntaxin monoclonal antibody (MAB 336). (b) densitometric analysis of synaptobrevin level in human brain cortex synaptosomes after TeTx exposure. Data are expressed as percentage of synaptobrevin in control synaptosomes. Each band of synaptobrevin was normalized to the corresponding syntaxin band. Protein content was analysed using a computerized densitometer. Bars are mean±s.e.mean of $n=3$ different blots. $*P<0.005$ when compared to the control value (two-tailed Student's *t*-test).

release was dependent on external Ca^{2+}, it was blocked when the Ca^{2+} chelator BAPTA was present in the cytoplasm and it was highly sensitive to the clostridial toxin TeTx. The overflow of DA evoked by 4-aminopyridine also was dependent on external Ca^{2+}, it was prevented by the VSCC blocker Cd^{2+} and it was abolished following TeTx internalization into nerve terminals.

Treatment of rat brain tissue preparations with low micromolar concentrations of Ca^{2+} ionophores has been reported to elicit external Ca^{2+}-dependent vesicular exocytosis, with no involvement of VSCCs (Sanchez Prieto *et al*., 1987; Verhage *et al*., 1991; Von Gersdorff & Matthews, 1994; Capogna *et al*., 1996; Lonart *et al*., 1998; Fassio *et al*., 1999). In our work, removal of Ca^{2+} from the superfusion medium abolished completely the releasing action of ionomycin, while the lack of effect of Cd^{2+} ions excludes VSCC activation. These results with human neocortex synaptosomes, together with recent findings showing that, in rat synaptosomes, ionomycin-induced release of unmetabolized DA and this release was insensitive to blockers of non-vesicular carrier-mediated DA release (Fassio *et al*., 1999), strengthen the idea that ionomycin can release DA from human neocortex by an exocytotic-like process.

Caffeine is known to cause Ca^{2+} mobilization from ryanodine-sensitive stores in the endoplasmic reticulum. According to Berridge (1998), caffeine mobilizes Ca^{2+} by enhancing the Ca^{2+} sensitivity of the RYRs; in turn, increasing the resting cytosolic level of Ca^{2+} greatly increase the sensitivity of RYRs to caffeine (Kano *et al*., 1995). Such a cooperativity could apparently generate the localized high concentration burst of Ca^{2+} necessary to trigger exocytosis. On the other hand, in spite of the evidence for caffeine-induced Ca^{2+} release in neurons, caffeine-induced transmitter release from brain tissue, excluding that indirectly mediated through blockade of adenosine receptors, has not been described. Caffeine was reported to elicit DA release from PC12 cells (Avidor *et al*., 1994; Koizumi & Inoue, 1998), although the mechanisms proposed appear contradictory. The first report excludes a major role for caffeine-mediated Ca^{2+} entry in the caffeine-activated DA secretion, whereas the second report claims that Ca^{2+} entry evoked by caffeine has an indispensable role in the secretory response. Our data with human nerve endings clearly show that the DA release evoked by caffeine was totally independent of external Ca^{2+}.

When human neocortex synaptosomes were pre-treated 8 min before caffeine with dantrolene, a drug thought to prevent Ca^{2+} release from ryanodine-sensitive stores, the overflow evoked by caffeine was significantly decreased, but only by about 50%. The caffeine-evoked overflow also was attenuated in synaptosomes entrapped with heparin, an antagonist at $InsP_3Rs$, suggesting that Ca^{2+} released into the cytoplasm by caffeine can affect the function of $InsP_3Rs$. This may occur through a kind of internal Ca^{2+}-induced Ca^{2+} release (CICR) process independent of VSCC activation (Zacchetti *et al*., 1991). Another possibility is that, as proposed by Hernández-Cruz *et al*. (1997), the RYRs (activated by caffeine) function to amplify the Ca^{2+} signals emanating from the $InsP_3$ receptors, thus contributing to trigger DA exocytosis. Whatever the mechanism, the complete block of the caffeine effect by the combined treatment with dantrolene and heparin favours the view that both RYRs and $InsP_3Rs$ cooperate in providing Ca^{2+} for DA exocytosis during caffeine treatment.

Synaptosomes isolated subsequent to homogenization of human neocortical tissue in presence of 500 nM TeTx were unable to release DA upon depolarization with either 15 mM K^+ or 100 μM 4-aminopyridine, indicating that enough toxin was incorporated to cleave all the synaptobrevin involved in the DA exocytosis triggered by these depolarizing stimuli. In all previous work with rat striatal synaptosomes incubated with TeTx for 90 min, the K^+-evoked overflow of DA could maximally be inhibited by only 40% (Fassio *et al*., 1999). The release of other transmitters from rat synaptosomes depolarized with high-K^+ also was only in part sensitive to clostridial toxin incubation (Habermann *et al*., 1988; McMahon *et al*., 1992; Ashton & Dolly, 1997). The reasons for the apparent higher sensitivity to TeTx of DA exocytosis from human mesocortical endings are at present unclear. It seems not to be due to a more homogenous cytoplasmic distribution of entrapped TeTx versus a localized distribution of TeTx internalized during incubation; in fact, the overflow of GABA and glutamate evoked by 15 mM KCl from rat cerebrocortical synaptosomes entrapped with 500 nM TeTx could be maximally inhibited by only 60% (Raiteri *et al*., 2000). As to the cleavage of synaptobrevin by TeTx, the toxin was shown to produce its maximal enzymatic effect ($\sim$50% cleavage) in rat synaptosomes prepared in the presence of 100 nM TeTx, while no larger cleavage could be seen with 500 nM TeTx (Raiteri *et al*., 2000). In human synaptosomes entrapped with 500 nM TeTx, synaptobrevin was $\sim$50% cleaved, as in rat synaptosomes. Although, due to the heterogeneity of the synaptosomal preparations, the extent of the cleavage of synaptobrevin in dopaminergic terminals, a small percentage of the total, is unknown, DA exocytosis evoked by depolarization from human mesocortical axon terminals seems particularly sensitive to TeTx.

The release of DA elicited by ionomycin and by caffeine, respectively dependent on external Ca^{2+} and on Ca^{2+} entering the cytoplasm from the endoplasmic reticulum, were completely insensitive to TeTx, suggesting the existence of exocytotic processes that, within the same neuronal system, differ depending on the triggering stimulus. At a first glance, depolarization and activation of VSCCs appear to be at the basis of the sensitivity to TeTx, while exocytotic release triggered by Ca^{2+} originating through mechanisms bypassing VSCC activation, although quantitatively comparable to that provoked by depolarization and VSCCs activation, can not be affected by TeTx. At present, the reasons for this striking difference in sensitivity to TeTx are unclear, but the existence of 'pools' of synaptobrevin having differential sensitivity to TeTx could be taken into consideration. One way by which part of synaptobrevin can be protected from cleavage is through its assembly in the SNARE complex (Hayashi *et al*., 1994; Pellegrini *et al*., 1994). Alternatively it was observed that toxin-cleaved SNARE components can still assemble into SNARE complexes, although of lower stability (Hayashi *et al*., 1995; Pellegrini *et al*., 1995); this decreased stability could affect exocytosis strictly linked to VSCC activation, while the more diffuse distributions of Ca^{2+} into the cytoplasm occurring after ionomycin or caffeine treatment could rescue exocytotic release (Sanchez-Prieto *et al*., 1987; Ashton & Dolly, 1991; Capogna *et al*., 1997). As a third possibility, the existence of a TeTx-resistant isoform of VAMP/synaptobrevin (Bock & Scheller, 1997; Verderio *et al*., 1999) supporting exocytosis independent of VSCC activation should be considered.

The ability of caffeine to stimulate DA release from human mesocortical nerve endings deserves some comments. A caffeine-stimulated DA release has previously only been observed in PC12 cells (Avidor *et al*., 1994; Koizumi & Inoue,

1998). Caffeine increased striatal DA release during *in vivo* microdialysis, but the effect was indirectly mediated through the known blocking activity of caffeine at adenosine receptors (Okada *et al*., 1997). The characteristics of the superfusion system used here to monitor release and the results with RYR and $InsP_3R$ blockers permit us to conclude that caffeine acts directly on human nerve terminals and causes DA release by mobilizing Ca^{2+} from the endoplasmic reticulum of mesocortical dopaminergic neurons. The release of DA in the neocortex might be involved in the purported cognition enhancing properties of caffeine.

This work was supported by an Italian Murst Network grant (1997). The authors thank Mrs Maura Agate for her skilful secretarial assistance.

References

ÅKERMAN, K.E.O. & HEINONEN, E. (1983). Qualitative measurements of cytosolic calcium ion concentration within isolated guinea pig nerve endings using entrapped arsenazo III. *Biochim. Biophys. Acta,* **732,** 117–121.

ASHTON, A.C. & DOLLY, J.O. (1991). Microtubule-dissociating drugs and A23187 reveal differences in the inhibition of synaptosomal transmitter release by botulinum neurotoxins types A and B. *J. Neurochem.,* **56,** 827–835.

ASHTON, A.C. & DOLLY, J.O. (1997). Microtubules and microfilaments participate in the inhibition of synaptosomal noradrenaline release by tetanus toxin. *J. Neurochem.,* **68,** 649–658.

AVIDOR, T., CLEMENTI, E., SCHWARTZ, L. & ATLAS, D. (1994). Caffeine-induced transmitter release is mediated via ryanodine-sensitive channel. *Neurosci. Lett.,* **165,** 133–136.

BERRIDGE, M.J. (1998). Neuronal calcium signalling. *Neuron,* **21,** 13–26.

BLÖCHL, A. & THOENEN, H. (1995). Characterization of nerve growth factor release from hippocampal neurons–evidence for a constitutive and an unconventional sodium-dependent regulated pathway. *Eur. J. Neurosci.,* **7,** 1220–1228.

BOCK, J.B. & SCHELLER, R.H. (1997). A fusion of new ideas. *Nature,* **387,** 133–135.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analyt. Biochem.,* **72,** 248–254.

CAPOGNA, M., GÄHWILER, B.H. & THOMPSON, S.M. (1996). Presynaptic inhibition of calcium-dependent and -independent release elicited with ionomycin, gadolinum, and α-latrotoxin in the hippocampus. *J. Neurophysiol.,* **75,** 2017–2028.

CAPOGNA, M., McKINNEY, R.A., O'CONNOR, V., GÄHWILER, B.H. & THOMPSON, S.M. (1997). Ca^{2+} or Sr^{2+} partially rescues synaptic transmission in hippocampal cultures treated with botulinum toxin A and C, but not tetanus toxin. *J. Neurosci.,* **17,** 7190–7202.

FASSIO, A., SALA, R., BONANNO, G., MARCHI, M. & RAITERI, M. (1999). Evidence for calcium-dependent vesicular transmitter release insensitive to tetanus toxin and botulinum toxin type F. *Neuroscience,* **90,** 893–902.

FINCH, E.A., TURNER, T.J. & GOLDIN, S.M. (1991). Calcium as a coagonist of inositol 1,4,5,-trisphosphate-induced calcium release. *Science,* **252,** 443–446.

FRANDSEN, A. & SCHOUSBOE, A. (1992). Mobilization of dantrolene-sensitive intracellular calcium pools is involved in the cytotoxicity induced by quisqualate and N-methyl-D-aspartate but not by 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate and kainate in cultured cerebral cortical neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 2590–2594.

FRIEL, D.D. & TSIEN, R.W. (1992). A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurons modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. *J. Physiol.,* **450,** 217–246.

HABERMANN, E., MÜLLER, H. & HUDEL, M. (1988). Tetanus toxin and botulinum A and C neurotoxins inhibit noradrenaline release from cultured mouse brain. *J. Neurochem.,* **51,** 522–527.

HAYASHI, T., McMAHON, H., YAMASAKI, S., BINZ, T., HATA, Y., SÜDHOF, T.C. & NIEMANN, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *Eur. Mol. Biol. Org. J.,* **13,** 5051–5061.

HAYASHI, T., YAMASAKI, S., NAUENBURG, S., BINZ, T. & NIEMANN, H. (1995). Disassembly of the reconstituted synaptic vesicle membrane fusion complex *in vitro*. *Eur. Mol. Biol. Org. J.,* **14,** 2317–2325.

HERNÁNDEZ-CRUZ, A., ESCOBAR, A.L. & JIMENEZ, N. (1997). Ca^{2+}-induced Ca^{2+} release phenomena in mammalian sympathetic neurons are critically dependent on the rate of rise of trigger Ca^{2+}. *J. Gen. Physiol.,* **109,** 147–167.

KANO, M., GARASCHUK, O., VERKHRATSKY, A. & KONNERTH, A. (1995). Ryanodine receptor-mediated intracellular calcium release in rat cerebellar Purkinje neurons. *J. Physiol.,* **487,** 1–16.

KOIZUMI, S. & INOUE, K. (1998). Functional coupling of secretion and capacitative calcium entry in PC12 cells. *Biochem. Biophys. Res. Commun.,* **247,** 293–298.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680–685.

LINK, E., EDELMANN, L., CHOU, J.H., BINZ, T., YAMASAKI, S., EISEL, U., BAUMERT, M. & SÜDHOF, T.C., NIEMANN, H. & JAHN, R. (1992). Tetanus toxin action: inhibition of neurotransmitter release linked to synaptobrevin proteolysis. *Biochem. Biophys. Res. Commun.,* **189,** 1017–1023.

LONART, G., JANZ, R., JOHNSON, K.M. & SÜDHOF, T.C. (1998). Mechanism of action of rab3A in mossy fiber LTP. *Neuron,* **21,** 1141–1150.

McMAHON, H.T., FORAN, P., DOLLY, J.O., VERHAGE, M., WIEGANT, V.M. & NICHOLLS, D.G. (1992). Tetanus toxin and botulinum type A and B inhibit glutamate, γ-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. *J. Biol. Chem.,* **267,** 21338–21343.

McPHERSON, P.S., KIM, Y.K., VALDIVIA, H., KNUDSON, C.M., TAKEORA, H., FRANZINI-ARMSTRONG, C., CORONADO, R. & CAMPBELL, K.P. (1991). The brain ryanodine receptor: a caffeine-sensitive calcium release channel. *Neuron,* **7,** 17–25.

OKADA, M., KIRYU, K., KAWATA, Y., MIZUNO, K., WADA, K., TASAKI, H. & KANEKO, S. (1997). Determination of the effects of caffeine and carbamazepine on striatal dopamine release by *in vivo* microdialysis. *Eur. J. Pharmacol.,* **321,** 181–188.

PARNESS, J. & PALNITKAR, S.S. (1995). Identification of dantrolene binding sites in porcine skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.,* **270,** 18465–18472.

PELLEGRINI, L.L., O'CONNOR, V. & BETZ, H. (1994). Fusion complex formation protects synaptobrevin against proteolysis by tetanus toxin light chain. *Fedn. Eur. Biochem. Soc. Lett.,* **353,** 319–323.

PELLEGRINI, L.L., O'CONNOR, V., LOTTSPEICH, F. & BETZ, H. (1995). Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion. *Eur. Mol. Biol. Org. J.,* **14,** 4705–4713.

PENG, Y.Y. (1996). Ryanodine-sensitive component of calcium transients evoked by nerve firing at presynaptic nerve terminals. *J. Neurosci.,* **16,** 6703–6712.

RAITERI, M., ANGELINI, F. & LEVI, G. (1974). A simple apparatus for studying the release of neurotransmitter from synaptosomes. *Eur. J. Pharmacol.,* **25,** 411–414.

RAITERI, M., SALA, R., FASSIO, A., ROSSETTO, O. & BONANNO, G. (2000). Entrapping of impermeant probes of different size into non-permeabilized synaptosomes as a method to study presynaptic mechanisms. *J. Neurochem.,* **74,** 423–431.

SANCHEZ-PRIETO, J., SIHRA, T.S., EVANS, D., ASHTON, A., DOLLY, J.O. & NICHOLLS, D.G. (1987). Botulinum toxin A blocks glutamate exocytosis from guinea-pig cerebral cortical synaptosomes. *Eur. J. Biochem.,* **165,** 675–681.

SCHIAVO, G., BENFENATI, F., POULAIN, B., ROSSETTO, O., POLVERINO DE LAURETO, P., DASGUPTA, B.R. & MONTECUCCO, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature,* **359,** 832–835.

SIMPSON, P.B., CHALLIS, R.A.J. & NAHORSKI, S.R. (1995). Neuronal Ca^{2+} stores: activation and function. *Trends Neurosci.,* **18,** 299–306.

SMITH, A.B. & CUNNANE, T.C. (1996). Ryanodine-sensitive calcium stores involved in neurotransmitter release from sympathetic nerve terminals of the guinea pig. *J. Physiol.,* **497,** 657–664.

TAYLOR, C.W. & RICHARDSON, A. (1991). Structure and function of inositol trisphosphate receptors. *Pharmacol. Ther.,* **51,** 97–137.

THAYER, S.A., HIRNING, L.D. & MILLER, R.J. (1988). The role of caffeine-sensitive calcium stores in the regulation of the intracellular free calcium concentration in rat sympathetic neurons in vivo. *Mol. Pharmacol.,* **34,** 664–673.

TSE, F.W., TSE, E., HILLE, B., HORSTMANN, H. & ALMERS, W. (1997). Local Ca^{2+} release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron,* **18,** 121–132.

VERDERIO, C., COCO, S., BACCI, A., ROSSETTO, O., DE CAMILLI, P., MONTECUCCO, C. & MATTEOLI, M. (1999). Tetanus toxin blocks the exocytosis of synaptic vesicles clustered at synapses but not of synaptic vesicles in isolated axons. *J. Neurosci.,* **19,** 6723–6732.

VERHAGE, M., McMAHON, H.T., GHIJSEN, W.E.J.M., BOOMSMA, F., SCHOLTEN, G., WIEGANT, V.M. & NICHOLLS, D.G. (1991). Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron,* **6,** 517–524.

VON GERSDORFF, H. & MATTHEWS, G. (1994). Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals. *Nature,* **367,** 735–739.

WATKINS, C.S. & MATHIE, A. (1996). Effects on K^+ currents in rat cerebellar granule neurones of a membrane-permeable analogue of the calcium chelator BAPTA. *Br. J. Pharmacol.,* **118,** 1772–1778.

WILLIAMSON, L.C., HALPERN, J.L., MONTECUCCO, C., BROWN, J.E. & NEALE, E.A. (1996). Clostridial neurotoxins and substrate proteolysis in intact neurons: botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. *J. Biol. Chem.,* **271,** 7694–7699.

ZACCHETTI, D., CLEMENTI, E., FASOLATO, C., LORENZON, P., ZOTTINI, M., GROHOVAZ, F., FUMAGALLI, G., POZZAN, T. & MELDOLESI, J. (1991). Intracellular Ca^{2+} pools in PC12 cells. A unique rapidly exchanging pool is sensitive to both inositol 1,4,5-trisphosphate and caffeine-ryanodine. *J. Biol. Chem.,* **266,** 20152–20158.

(Received November 16, 1999
Accepted January 24, 2000)