groups which were exposed to different DHE levels was seen (range 151,745 ± 1710–167,594 ± 2060 cells/well). Examination of cells by phase contrast microscopy revealed no changes that could be ascribed to DHE.

Smooth muscle cell growth (table). Initial seeding density of smooth muscle was 7613 ± 176 cells/well. Plating efficiency measured at day 3 did not differ markedly being 5865 ± 380–5976 ± 412. By day 7 after seeding, the cell count had increased, with control wells containing 9017 ± 246 cells and DHE treated wells between 8872 ± 332 and 9051 ± 288 cells. Phase contrast microscopy revealed no changes in cell configuration.

Endothelial cell prostanoid production. The 24-h incubated media had concentrations of 6-keto $PGF_{1\alpha}$ of 3380 ± 380 pg/ml in controls, and a range of 3160 ± 425–3370 ± 510 pg/ml in DHE wells. TxB_2 levels were 5810 ± 1020 pg/ml in controls and ranged from 4210 ± 1400 to 6960 ± 1310 pg/ml in DHE wells. No significant differences existed between control medium and that to which DHE had been added.

Discussion. In the current study we could not verify increased activity of prostanoids in vitro from cultured endothelial cells exposed to DHE. We have reported previously on the effects in vivo on healthy volunteers of indomethacin pretreatment before DHE administration. After pretreatment the same hemodynamic effects were observed as with no pretreatment; namely, increased venous flow velocity, reduced venous cross-sectional area, and reduced resting calf blood flow volume[6, 10]. These results, contrary to those of others, suggest little importance of prostanoids in the action of DHE. Measured under identical conditions, other agents show substantial variation in the prostanoid activity. Reduced release of prostanoids has been noted for acetylsalicylic acid (– 55%) and other cyclooxygenase and thromboxane inhibitors, whereas dipyridamole, a phosphodiesterase inhibitor, did not alter prostanoid activity, and thrombin administration increased prostanoid activity[11].

In addition to the direct vascular effects of DHE, metabolic alterations have also been ascribed to DHE. Included among these are reductions in liver glucose output, inhibition of adrenaline-induced hyperglycemia, stimulation of lipolysis, inhibition of cAMP degradation, interference with phosphodiesterase and increased pyruvate/lactate ratio during relative hypoxia[7, 12]. Because of the potential effect of these changes on cell growth, we attempted to define whether there was any interaction between DHE and in vitro endothelial or smooth muscle plating efficiency and cell proliferation. Our data show that no such effect exists in this setting.

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Inhibition by tetanus and botulinum A toxin of the release of [^{3}H]noradrenaline and [^{3}H]GABA from rat brain homogenate

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Summary. Rat brain homogenate was preloaded with [^{3}H]noradrenaline or [^{3}H]GABA and stimulated with high K^+. Tetanus toxin and botulinum A neurotoxin partially prevent the evoked [^{3}H]noradrenaline release in the same range of toxin concentrations starting below 10^{-10} M. In contrast, release of γ-amino butyric acid (GABA) is much more sensitive to tetanus than to botulinum A toxin.
Key words. Tetanus toxin; botulinum toxin; noradrenaline; GABA; brain.

Recently it has been claimed[1] that tetanus toxin does not inhibit the evoked release of [^{3}H]noradrenaline from rat cerebral cortex slices. The statement contradicts our positive findings, obtained using two different experimental designs, on rat brain homogenate[2, 3]. Using an improved superfusion system, we now show that both tetanus and, still better, botulinum A toxin prevent noradrenaline release. In contrast, the release of GABA, which is known as an inhibitory neurotransmitter, is inhibited preferentially by tetanus toxin.

Method. Rat brain cortex was cut into 0.3 × 0.3 mm prisms with a McIlwain chopper and suspended in Hepes (10 mM, pH 7.4) buffered ice-cold Krebs-Ringer solution (KRH). After a short trituration with an Eppendorf pipette, the suspension (10% w/v) was passed three times by hand through a Potter homogenizer with a loose-fitting pestle. After 3 min centrifugation at 4500 × g and washing once, the sediment was taken up to give 2% (w/v) with respect to original weight. For studying noradrenaline release, this suspension was mixed with half its volume of KRH containing (final concentration) noradrenaline (1 mCi/l, 1 μM), iproniazid (10 μM) and ascorbic acid (20 μM). All subsequent solutions contained iproniazid and ascorbic acid. After 15 min shak-

ing at 37 °C, the preloaded homogenate was centrifuged. The sediment was twice resuspended in buffer and centrifuged, and finally made up to 2% (w/v) in KRH. For studies of GABA release, labeled and unlabeled noradrenaline were replaced by the respective GABA concentrations, whereas aminooxyacetic acid (10^{-5} M) was substituted for iproniazid and ascorbic acid. Final [^{3}H]GABA concentration was 3 mCi/l.

The prelabeled suspension (0.2 ml) was mixed with the neurotoxins in 0.1 ml KRH containing 1 mg/ml bovine serum albumin, together with the stabilizers mentioned. After 2 h at 37 °C, the samples were filled into Millipore Swinnex filter holders (13 mm diameter, 12 holders) over a double layer of Whatman GF-C glass fiber filters. After 15 min superfusion at 37 °C with 0.5 ml/min KRH, 1-min fractions were collected for liquid scintillation counting. The K^+ content of KRH was then increased from 5.9 to 31 mM by equimolar substitution of KCl for NaCl. A 2-min pulse released between 35 and 50% of the radioactivity, depending on the preparation. The remainder was quantitatively eluted by final superfusion with 0.5 M acetic acid (fig. 1). Total content in radioactivity, absolute and fractional release were calculated. Each figure represents data from a single experiment; each experiment was repeated at least twice with essentially the same result. It was confirmed that tetanus or botulinum A toxin did not change the velocity of [^{3}H]noradrenaline uptake[2,3], that the K^+-concentration selected was suboptimal, that neuraminidase treatment did not influence the effect of tetanus toxin whereas antibodies neutralized it[2], and that the released radioactivity behaved nearly exclusively like noradrenaline in chromatography[4].

Quadruplicate samples without and with 167 ng/ml (1.1×10^{-9} M) tetanus toxin were run under the described conditions to assess intraassay standard deviations (SD) and statistical significance. Evoked release when given in % was 38 ± 1.9, and 20.1 ± 2.1 ($p < 0.01$). When given in Bq, it was 80.3 ± 7.7, and 46.3 ± 6.6 ($p < 0.01$). Total content was 196.2 ± 4.7, and 227.9 ± 13.4 ($p > 0.01 < 0.05$).

Tetanus toxin (LD_{50}, mice, 2 ng/kg) was prepared in this laboratory. Botulinum A neurotoxin (LD_{50} 1 ng/kg, free from hemagglutinin) was from Dr Frevert, Battelle-Institut, Frankfurt, FRG.

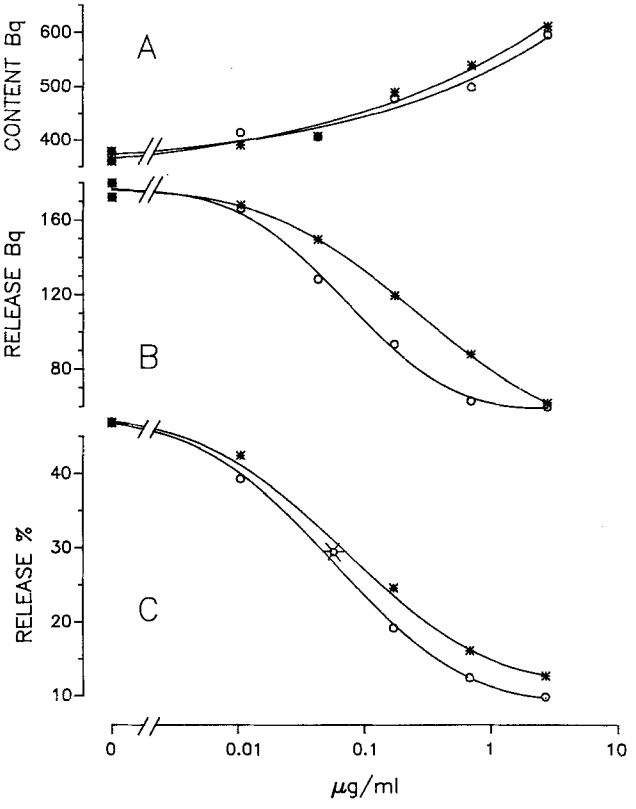

Figure 2. Inhibition of K^+-evoked release of [^{3}H]noradrenaline by tetanus (*) and botulinum A toxin (○): Increase in total content (*A*), and decrease of absolute (*B*) and fractional (*C*) release. Toxin-free controls (■) were run in duplicate, whereas the other data points represent single values. *A* gives the total content of radioactivity (Bq/sample) at the start of depolarization. *B* shows the K^+-evoked release (Bq) (see fig. 1), corrected for the basal release. *C* represents the same data, but used to calculate fractional release. The abscissa gives the final toxin concentrations.

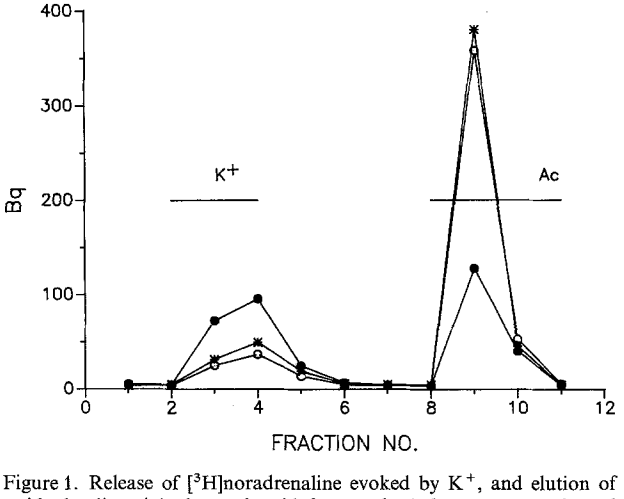

Figure 1. Release of [^{3}H]noradrenaline evoked by K^+, and elution of residual radioactivity by acetic acid, from rat brain homogenate poisoned with tetanus toxin (*), botulinum A neurotoxin (○, both 0.67 µg/ml), and from a toxin-free control (●).

The pretreated and prewashed (see methods) preparations were superfused with 31 mM K^+ (fract. 3 and 4) and acetic acid (fract. 9–11). The efflux is given as Bq/sample (ordinate). Total content was the sum of radioactivity in fract. 3–11, and evoked release was the sum of fract. 3–5. Each data point in the figures refers to a single measurement. All experiments were repeated at least twice with essentially the same result.

Results. Both tetanus and botulinum A toxin partially prevent the [^{3}H]noradrenaline release (fig. 1). According to the concentration-response curves (fig. 2) the detection limit for the toxins was 10 ng/ml, corresponding to 67 pM. Even with the highest toxin concentrations (2×10^{-8} M) inhibition of evoked release was incomplete. Over the comparable dose range, botulinum A toxin was about twice as potent as tetanus toxin (fig. 2C).

Because of their variability in thickness and weight, work with slices can only furnish relative values, i.e. fractional release[1]. In contrast, the low intraassay variation of our particulate preparation (see Methods) allows us to compare absolute values of total content (fig. 2A) and release (fig. 2B) in terms of radioactivity. Both tetanus and botulinum A toxin increased the total content of radioactivity present at the start of depolarization. Since uptake was not enhanced[2,3], the higher total content (fig. 2A) must have been due to a decreased basal release during the incubation[3] and the thorough washing that precedes the depolarization. On the other hand, evoked release is also inhibited when given in absolute terms (fig. 2B). Thus the eventual change in fractional release (fig. 2C) results from the opposite effects of the toxins on content and release of radioactivity.

To provide a comparison with previous work[1] we have replaced the homogenate by suspended slices (0.5×0.5 mm,

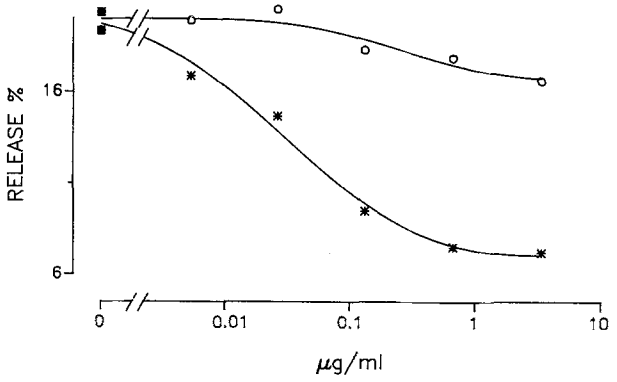

Figure 3. Inhibition of the K^+-evoked [^{3}H] GABA release by tetanus as compared with botulinum A toxin. Ordinate and abscissa as in figure 2C. (■) Toxin-free controls.

2% w/v) under otherwise identical conditions. Tetanus toxin and botulinum A toxin (both 1.67 μg/ml) reduced the fractional evoked release from 25.5 to 11 and 4.5%, respectively. Ten-fold lower concentrations of tetanus toxin were no longer active (22.5%), whereas botulinum A toxin still was (15.5%, all values are the means of duplicates). Throughout their work Heredero and Oja [1] used one single tetanus toxin concentration (1.5×10^3 MLD/ml). This concentration (about 75 ng/ml as calculated from an LD_{50} of 2 ng/kg) was too low to have an effect on our slices, too, but was clearly effective (see fig. 2) on brain homogenate. The finding demonstrates again a disadvantage of slices for studying tetanus toxin action on transmitter release.

Tetanus toxin also inhibits the release of [^{3}H]GABA (fig. 3). In contrast to noradrenaline release, botulinum toxin was slightly active, but one to two orders of magnitude less potent than tetanus toxin. Moreover, neither toxin led to an accumulation of [^{3}H]GABA (not shown), in contrast to [^{3}H]noradrenaline (see figs 1 and 2).

The data corroborate our previous finding [2] that tetanus and botulinum A toxin inhibit both noradrenaline and GABA release. Although botulinum A toxin can ascend to the spinal cord [6], its very low potency as an inhibitor of GABA release explains why this toxin does not share the tetanic action in vivo. The slightly higher inhibitory potency of botulinum A as compared with tetanus toxin on noradrenaline release fits in with our recent observation on primary nerve cell cultures from rat brain cortex. Here botulinum A toxin was also more potent than tetanus toxin [5]. In our previous experiments [2] botulinum A toxin had been less potent than tetanus toxin in inhibiting noradrenaline release. The difference may be due to the superior purity and preservation of the presently available botulinum A neurotoxin.

Currently we are using inhibition of noradrenaline release from superfused brain homogenate to measure the in vitro activity of all clostridial neurotoxins or their fragments. The system also furnishes functional in vitro data for the titration of antibodies.

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Effect of AD6 (8-monochloro-3-beta-diethylamino-ethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin) on cyclic nucleotide phosphodiesterases in human platelets

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Summary. The effect of AD6 (8-monochloro-3-beta-diethylamino-ethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin), an inhibitor of platelet aggregation, on cyclic nucleotide metabolism was investigated. AD6 inhibited selectively human platelet cyclic GMP phosphodiesterase, which was separated from cyclic AMP phosphodiesterase by DEAE-cellulose chromatography. Addition of AD6 to washed platelets increased cyclic GMP levels significantly in two minutes. These results could be useful in elucidating the action of AD6 on intact platelets.
Key words. AD6; cyclic GMP; cyclic nucleotide phosphodiesterase inhibition; platelets.

Cyclic AMP is clearly implicated in platelet function, acting as a negative modulator and possibly affecting the intracellular calcium homeostasis [1]. An increase in cyclic AMP levels causes an inhibition of platelet aggregation, whereas agents reducing cyclic AMP levels lead to or potentiate platelet aggregation. On the other hand, the role of cyclic GMP remains controversial, since both aggregating and disaggregating effects have been described [2]. Although cyclic GMP has been reported to increase in human platelets stimulated by thrombin or collagen [1], it has been proposed recently that cyclic GMP could exert inhibitory effects on platelet activation [3]. This effect has been related to calcium mobilization, both through the plasma membrane and from internal stores [4].

Cyclic nucleotides may be elevated intracellularly in a selective manner, through the action of phosphodiesterase (PDE) inhibitors [5]. Some inhibitors, like RO 15-2041, do not increase cyclic nucleotide levels at a concentration which affect