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Protein kinase C activation and α_2-autoreceptor-modulated release of noradrenaline

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1 Effects of phorbol esters on the evoked noradrenaline release were studied in slices of the rabbit hippocampus, labelled with [^{3}H]-noradrenaline, superfused continuously with a medium containing the reuptake inhibitor cocaine and stimulated electrically for 2 min (stimulation parameters: 2 ms, 24 mA, 5 V cm^{-1}, 3 or 0.3 Hz).

2 The electrically-evoked overflow of [^{3}H]-noradrenaline in the slices was increased in a concentration-dependent manner by the protein kinase C (PKC) activators 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and 4β-phorbol 12,13-dibutyrate (4β-PDB). Phorbol esters, which do not activate PKC, 4-*O*-methyl-TPA and 4α-PDB, showed no effect on neurotransmitter release. The effect of 4β-PDB was abolished in the presence of tetrodotoxin and in the absence of calcium. The PKC inhibitor polymyxin B inhibited the evoked noradrenaline release.

3 In the presence of 4β-PDB the inhibitory effects of the α_2-adrenoceptor agonist clonidine or the facilitatory effects of the α_2-adrenoceptor antagonist yohimbine seemed to be modified only by changes in the concentration of noradrenaline in the synaptic region. At a stimulation frequency of 3 Hz the inhibitory action of clonidine was reduced whereas the facilitatory effect of the yohimbine was even slightly enhanced by the phorbol ester. At 0.3 Hz and in the presence of 4β-PDB the effect of clonidine remained and that of yohimbine was strongly enhanced.

4 Pretreatment of the slices with islet-activating protein or N-ethylmaleimide significantly reduced the enhancement of noradrenaline release caused by 4β-PDB. It is possible that a regulatory N-protein is involved in steps following PKC activation.

5 These results suggest that PKC participates in the mechanism of action-potential-induced noradrenaline release from noradrenergic nerve terminals of the rabbit hippocampus and that effects on the autoinhibitory feedback system were not responsible for the 4β-PDB-induced increase of neurotransmitter release.

Introduction

In brain the existence of two regulatory N-proteins has been demonstrated (Sternweis & Robishaw, 1984). One of them seems to be identical to N_i, mediating receptor-coupled inhibition of adenylate cyclase, whereas the function of the other N-protein, N_o, is unknown. The α-subunits of both proteins are ADP-ribosylated by islet-activating protein (Sternweis & Robishaw, 1984; Asano & Ogasawara, 1986) or alkylated by N-ethylmaleimide (Asano & Ogasawara, 1986) and the ability of these proteins to couple the receptor with its effector system is inhibited (Jakobs *et al.*, 1984; Asano & Ogasawara, 1986). Recently, we demonstrated that pretreatment of rabbit hippocampal slices with either islet-activating protein or N-ethylmaleimide reduced both the inhibitory actions of the α_2-adrenoceptor agonist clonidine or of endogenous noradrenaline and the facilitatory effect of the α_2-adrenoceptor antagonist yohimbine on the electrically-evoked noradrenaline release (Allgaier *et al.*, 1985; 1986a). These results indicate the possible involvement of a regulatory N protein in the α_2-autoinhibitory feedback system (Allgaier *et al.*, 1985; 1986a). We concluded from earlier results obtained with adenylate cyclase activators, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) analogues or phosphodiesterase inhibitors (Cubeddu *et al.*, 1975; Stjärne *et al.*, 1979; Schoffelmeer & Mulder, 1983; Markstein *et al.*, 1984) that the N-protein participating in the autoinhibitory feedback

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mechanism may be identical to N_1 (Allgaier *et al.*, 1985; 1986a).

In intact platelets and in cyc$^-$ S49 lymphoma cells the N_i-mediated inhibition of adenylate cyclase by hormonal factors was reduced or even abolished by the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Jakobs *et al.*, 1985; Katada *et al.*, 1985). Activation of PKC by TPA in platelet membranes led to phosphorylation of the α-subunit of N_i (Katada *et al.*, 1985) suggesting that this is the mechanism whereby the receptor-coupled inhibition of adenylate cyclase was diminished (Katada *et al.*, 1985). In this regard the action of TPA resembles that of islet-activating protein or N-ethylmaleimide. Recently, we demonstrated that phorbol esters similar to islet-activating protein or N-ethylmaleimide enhanced noradrenaline release from noradrenergic nerve terminals of the rabbit hippocampus (Allgaier & Hertting, 1986; Allgaier *et al.*, 1986b). Hence, the question arises as to whether activation of PKC may lead to an inactivation of the α_2-autoinhibitory feedback system of noradrenergic nerve terminals.

Methods

Superfusion experiments

These were performed according to the methods Jackisch *et al.* (1984) and Allgaier *et al.* (1985). Rabbits of either sex weighing about 2 kg were killed by decapitation. The brain was rapidly removed and dissected on a chilled plate. Slices (0.35–0.4 mm thick, 5–7 mg wet weight) of the middle third part of the hippocampus were prepared using a McIlwain tissue chopper in a direction corresponding to the lamellar organization of the hippocampus. The slices included the gyrus dentatus and the fimbria. They were washed and then incubated in 2 ml of a modified Krebs-Henseleit medium (composition in mM: NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, Na_2EDTA 0.03; saturated with 95% O_2 and 5% CO_2, pH 7.4) containing 0.1 μmol l^{-1} [^{3}H]-noradrenaline (44.4 Ci mmol^{-1}) for 30 min at 37°C. Subsequently the slices were washed, transferred to superfusion chambers (1 slice per chamber) and superfused at a rate of 1 ml min^{-1} with medium containing 30 μM cocaine. During superfusion the slices were stimulated twice electrically (stimulation parameters: rectangular pulses; 2 ms, 24 mA, 5 V cm^{-1}) after 60 (S_1) and 125 min (S_2) for 2 min each; routinely the stimulation frequency was 3 Hz (= 360 pulses 2 min^{-1}), in some experiments stimulation was performed at 0.3 Hz (= 36 pulses 2 min^{-1}). Total duration of superfusion was 150 min. Drugs or the respective solvent were added to the superfusion medium from 15 min before S_2 onwards in order to test their effects on noradrenaline release. In all experiments cocaine 30 μM was present throughout superfusion; 4β-phorbol 12,13-dibutyrate (4β-PDB; 1 μM) was present during superfusion as indicated. At the end of superfusion, the slices were removed from the chambers and dissolved in Soluene 350 (Packard Instrument, Frankfurt, FRG) before determination of tritium content.

Pretreatment of slices with islet-activating protein

In some experiments slices of the rabbit hippocampus (0.35 mm) were pretreated with islet-activating protein (IAP) before labelling with [^{3}H]-noradrenaline (see Allgaier *et al.*, 1985). Pretreatment was performed for 18 h at 37°C in 20 ml medium either in the presence of islet-activating protein (dissolved in NaCl 0.5 M, $Na_4P_2O_7$ 0.1 M, pH 7) or its solvent (controls) in an atmosphere of 95% O_2/5% CO_2 in culture dishes under gentle horizontal rotation. Further experimental protocol as described above.

Pretreatment of the slices with N-ethylmaleimide

N-ethylmaleimide treatment of the slices was performed before labelling with [^{3}H]-noradrenaline (Allgaier *et al.*, 1986a; 1987). Slices were preincubated for 30 min at 37°C in 40 ml medium containing N-ethylmaleimide (30 μM). Controls were preincubated with medium under the same conditions.

Calculation of release data

The fractional rate of tritium outflow (5 min)$^{-1}$ was calculated as tritium outflow per 5 min/tritium content in the slice at the start of the respective 5 min period (Hertting *et al.*, 1980; Allgaier *et al.*, 1987). The stimulation-evoked overflow of tritium was calculated by subtraction of the basal outflow from the total outflow of tritium. The basal outflow was assumed to decline linearly from the fraction 5 min before to the fraction 20–25 min after the onset of stimulation. The evoked-overflow of tritium (S_1, S_2) was expressed as % of the tritium content of the slice at the onset of the respective stimulation period. Effects of the various phorbol esters on noradrenaline release were evaluated by calculating the ratio S_2/S_1 of the overflow evoked by the two stimulation periods. The ratio S_2/S_1 as an estimate of drug effects was not used when effects of the α_2-adrenoceptor agonist clonidine or the α_2-adrenoceptor antagonist yohimbine on noradrenaline release were examined in slices either pretreated with IAP or NEM or superfused with 4β-PDB, since under these conditions the stimulation-evoked overflow was already strongly increased at S_1. In this case the differences $S_2 - S_1$ were used to illustrate drug-dependent changes in the absolute amount of the electrically

evoked tritium overflow. All results are shown as arithmetic means ± s.e.mean. The significance of differences between the treated groups was determined by use of Student's *t* test (two-tailed) or the Mann-Whitney test.

Chemicals

Drugs used were (−)-[ring-2,5,6-^{3}H]-noradrenaline (NEN, Dreieich, FRG); cocaine HCl and clonidine HCl (Boehringer, Ingelheim, FRG); yohimbine HCl (Merck, Darmstadt, FRG); polymyxin B sulphate (Serva, Heidelberg, BRD); 4β-phorbol-12, 13-dibutyrate (4β-PDB), 12-*O*-tetradecanoylphorbol-13-acetate (TPA); 4-*O*-methyl-TPA, N-ethylmaleimide and tetrodotoxin (Sigma, München, FRG); 4α-PDB (Paesel, Frankfurt, FRG). Stock solutions of the phorbol esters were made in dimethylsulphoxide (10 mM).

A concentrated sample of *Bordetella pertussis* toxin was obtained by citric acid precipitation of supernatants of *Bordetella pertussis* cultures, Tohama phase I strain, and redissolved in NaCl 0.5 M, $Na_4P_2O_7$ 0.1 M, pH 7. The content of islet-activating protein of the concentrated sample of *Bordetella pertussis* toxin was calculated by haemagglutination and leukocytosis-promoting assay as described previously (Allgaier *et al.*, 1985).

Results

Phorbol ester effects on noradrenaline release

Slices of the rabbit hippocampus were labelled with tritiated noradrenaline, superfused continuously and stimulated electrically (3 Hz, 2 ms, 24 mA, 5 V cm^{-1}) twice during the superfusion period (Figure 1). The effects of the PKC activator 4β-PDB (1 μM) and the PKC inhibitor polymyxin B (100 μM) on basal and on stimulation evoked outflow are shown in Figure 1a. The overflow of tritium evoked by the first stimulation (S_1) was about 4% of tissue tritium in drug-free medium and was strongly increased by addition of 4β-PDB and decreased by polymyxin B. In Figure 1b and c the effects of both drugs on the stimulation evoked overflow are depicted in two ways: either as changes in the S_2/S_1 ratios (Figure 1b) or as changes (decreases or increases) in the absolute amounts of evoked tritium ($S_2 - S_1$) with respect to the overflow of tritium evoked at S_1 (Figure 1c). Whereas the first type of presentation is most commonly used in neurotransmitter release experiments, the second type may be preferable when drug effects at different concentrations ('biophase concentrations') of endogenous agonists are to be compared. This latter type of presentation was chosen in some of the following figures (Figures 3–5).

The concentration-dependence of the increase of the evoked tritium overflow caused by 4β-PDB and TPA, another phorbol ester which activates PKC, is illustrated in Figure 2. The effect of 4β-PDB was much more pronounced: 4β-PDB 1 μM increased the evoked tritium overflow by more than 2 fold, whereas TPA, even at a concentration of 30 μM, caused only a facilitation of about 40%, as compared to the corresponding control values (obtained in the presence of dimethylsulphoxide). The basal tritium outflow remained unchanged by the phorbol esters up to a concentration of 10 μM 4β-PDB or 30 μM TPA. The solvent of the phorbol esters, dimethylsulphoxide, was at concentrations $< 0.3\%$ without effect both on basal and on stimulation-evoked tritium outflow. At a concentration of 0.3%, dimethylsulphoxide (used when the phorbol esters were administered at 30 μM) caused a slight but significant ($P < 0.05$) enhancement of the evoked tritium overflow of about 10%.

In order to investigate whether the ability of 4β-PDB and TPA to enhance the evoked tritium overflow was due to activation of PKC, the effects of 4α-PDB and 4-*O*-methyl-TPA, which are inactive on PKC (Gschwendt *et al.*, 1984; Ashendel 1985), were also examined. As shown in Figure 2, even high concentrations of both compounds did not enhance the electrically-evoked tritium overflow as compared to the respective dimethylsulphoxide controls.

In another series of experiments the Ca^{2+}-dependence and the sensitivity of the 4β-PDB-induced enhancement of the electrically evoked overflow to tetrodotoxin were studied. Hippocampal slices were superfused from 15 min before S_2 onwards with Ca^{2+}-free or tetrodotoxin-containing (0.3 μM) medium. The electrically evoked overflow of tritium was almost completely blocked in the absence of Ca^{2+} or in the presence of tetrodotoxin, irrespective of whether 4β-PDB (1 μM) was present or not (Table 1).

Effects of 4β-phorbol 12,13-dibutyrate on the action of clonidine and yohimbine on noradrenaline release

The enhancement of the evoked tritium overflow caused by 4β-PDB may be due to a functional impairment of the autoinhibitory feedback system. Therefore, the effects of the α_2-adrenoceptor agonist clonidine (Figure 3) and the α_2-adrenoceptor antagonist yohimbine (Figure 4) on the stimulation-evoked tritium overflow from hippocampal slices superfused throughout either with phorbol ester-free (Figures 3 and 4: open columns) or phorbol ester containing (Figures 3 and 4: hatched columns) medium were studied. Electrical field stimulation was performed at a frequency of 3 Hz (Figures 3a, 4a) or at 0.3 Hz (Figures 3b, 4b). The effects of clonidine and yohimbine on stimulation-evoked tritium overflow are depicted as $S_2 - S_1$ values as explained in Figure 1c.

At 3 Hz and in the absence of 4β-PDB the overflow

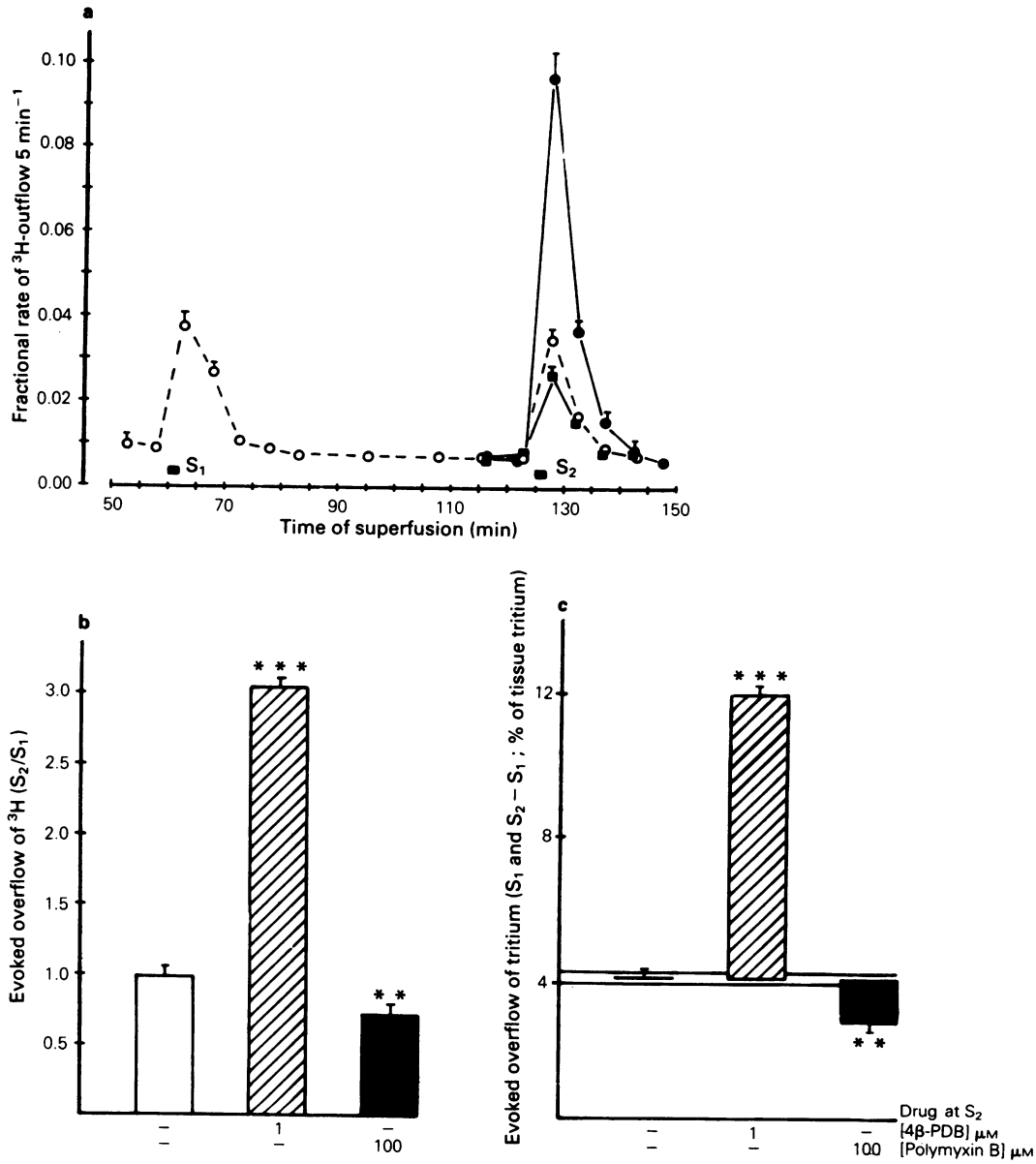

Figure 1 Effect of 4β-phorbol 12,13-dibutyrate (4β-PDB) and polymyxin B on basal and on electrically-evoked outflow of tritium from slices of the rabbit hippocampus, labelled with [^{3}H]-noradrenaline and then superfused continuously. Cocaine (30 μM) was present throughout superfusion. During superfusion slices were stimulated electrically twice at 60 (S_1) and 125 min (S_2) for two min each (2 ms, 24 mA, 5 Vcm^{-1}, 3 Hz). (a) Fractional rates of tritium outflow are depicted as means with s.e.mean shown by vertical bars (control (○), 4β-PDB (●), polymyxin B (■)). (b) The means of the ratios of the tritium overflow evoked by the two stimulation periods (S_2/S_1) are given (control: open column; 4β-PDB: hatched column; polymyxin B: solid column; vertical lines show s.e.mean. The evoked overflow of tritium is expressed as % of tritium content at the onset of the respective stimulation period. The mean of S_1 of all experiments was 4.10 ± 0.15 ($n = 16$). (c) S_1 ± s.e.mean of all experiments ($n = 16$) is depicted by a horizontal bar, $S_2 - S_1$ (expressed as % of tissue tritium) for the respective groups ($n = 4-6$) are depicted by vertical columns; vertical lines show s.e.mean. Significant differences from control: $^{**}P < 0.01$; $^{***}P < 0.001$.

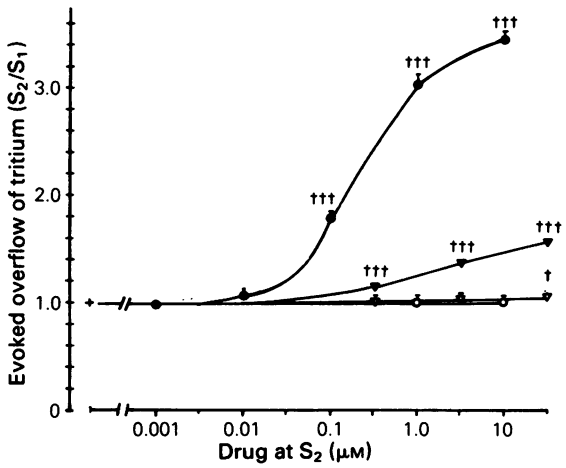

Figure 2 Effects of phorbol esters on the electrically-evoked overflow of tritium from slices of the rabbit hippocampus. Slices were prelabelled with [^{3}H]-noradrenaline, superfused continuously and stimulated electrically twice (3 Hz, 2 ms, 24 mA, 5 V cm^{-1}). 4β-phorbol 12, 13-dibutyrate (4β-PDB; ●), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; ▼), 4α-PDB (○) or 4-*O*-methyl-TPA (▽) were added to the superfusion medium from 15 min before S_2 onwards. Cocaine (30 μM) was present in all experiments throughout superfusion. Drug effects on the stimulation-evoked overflow are expressed as the ratio S_2/S_1 of the overflow of tritium evoked by the two stimulation periods. Means of the ratios S_2/S_1 are given; vertical lines show s.e.mean. Number of experiments: 6–9. Significant differences from control: †$P < 0.05$; †††$P < 0.001$. The significant enhancement of the evoked tritium overflow in the presence of 30 μM 4-*O*-methyl-TPA is due to the solvent dimethylsulphoxide.

evoked by the first electrical stimulation was about 4% of tissue tritium (Figures 3a and 4a: open horizontal bars). 4β-PDB, 1 μM, increased the evoked overflow of tritium by more than 2 fold (Figures 3a, 4a: hatched horizontal bars). In the presence of the phorbol ester (throughout superfusion) S_2 was always slightly decreased.

It is evident from Figure 3a, that the inhibitory action of clonidine on the electrically evoked overflow ($S_2 - S_1$, represented by vertical columns in the downward direction), with respect to either the clonidine-dependent changes in the absolute amount of evoked tritium or the effects of the agonist related to S_1, was strongly reduced or even abolished in the presence of 1 μM 4β-PDB. In contrast, the yohimbine-induced increase of the stimulation-evoked tritium overflow, which is due to blockade of the autoreceptor for endogenous noradrenaline in the vicinity of the axon terminals ('biophase' concentration of noradrenaline), remained unchanged or was even enhanced in the presence of 4β-PDB at a stimulation frequency of 3 Hz (Figure 4a: $S_2 - S_1$; vertical columns in upward direction). Only if the yohimbine-induced enhancement of the evoked tritium overflow was related to the respective S_1 value, was the antagonistic effect reduced by the phorbol ester.

At a stimulation frequency of 0.3 Hz (Figures 3b and 4b) the evoked overflow of tritium at S_1 – and hence the 'biophase' concentration of endogenous noradrenaline during stimulation – was strongly decreased to about 1% of tissue tritium (Figure 3b and 4b; open horizontal bars). Under these conditions, the inhibitory action of clonidine (Figure 3b: open vertical columns) was increased and the facilitatory effect of yohimbine (Figure 4b: open vertical columns) decreased as compared to the results obtained in

Table 1 Effect of 4β-phorbol 12,13-dibutyrate (4β-PDB) on the tritium overflow evoked by electrical stimulation in the absence of Ca^{2+} or in the presence of tetrodotoxin (TTX)

4β-PDB (1 μM)	*Ca^{2+}* (1.3 mM)	*TTX* (0.3 μM)	*S_2/S_1*	*Basal outflow*	n
–	+	–	1.02 ± 0.02	0.78 ± 0.02	6
+	+	–	3.05 ± 0.02	0.79 ± 0.02	6
–	–	–	0.02 ± 0.03	0.81 ± 0.02	4
+	–	–	0.02 ± 0.03	0.79 ± 0.02	4
–	+	+	0.05 ± 0.03	0.72 ± 0.02	4
+	+	+	0.16 ± 0.03	0.73 ± 0.02	4

Hippocampal slices, prelabelled with [^{3}H]-noradrenaline, were superfused in the presence of cocaine (30 μM) and stimulated electrically (3 Hz, 2 ms, 24 mA, 5 V cm^{-1}) twice. 4β-PDB was administered from 15 min before S_2 onwards in the presence or absence of Ca^{2+} (Ca^{2+} removal from the medium from 15 min before S_2 onwards) and in the presence of TTX (added 15 min before S_2). Effects on the stimulation-evoked overflow are expressed by the ratio S_2/S_1 of the overflow evoked by the two stimulation periods. Basal outflow is expressed as ratio between the fractional rate immediately before S_2 (120–125 min) and the fractional rate immediately before S_1 (55–60 min). Means ± s.e.mean of *n* observations are presented.

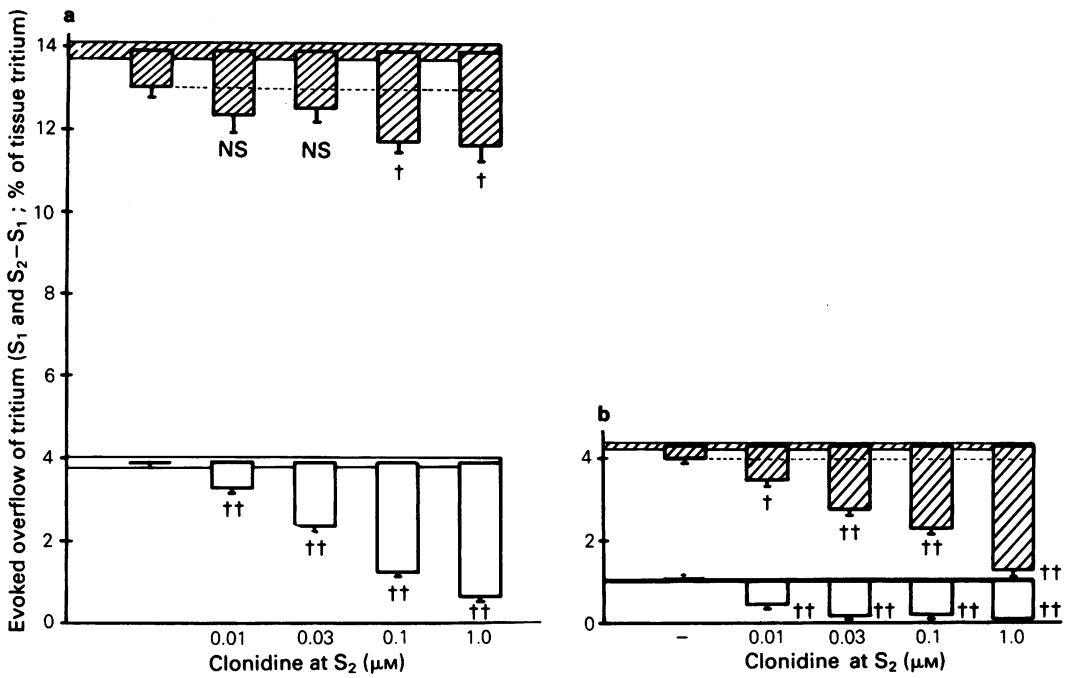

Figure 3 Effects of clonidine on the evoked overflow of tritium in the absence (open columns/bars) or the presence (hatched columns/bars) of 4β-phorbol 12,13-dibutyrate (4β-PDB, 1 μM; throughout superfusion). Clonidine was added to the superfusion medium from 15 min before S_2 onwards. Cocaine (30 μM) was present in all experiments throughout superfusion. The horizontal bars represent $S_1 \pm$ s.e.mean (expressed as % of tissue tritium) of all experiments either in the presence or the absence of 4β-PDB. $S_2 - S_1$ (expressed as % of tissue tritium) are represented by vertical columns; vertical lines show s.e.mean. The horizontal broken lines represent $S_2 - S_1$ of control. Stimulation was performed either at a stimulation frequency of 3 Hz (a) or of 0.3 Hz (b). Number of experiments of each group: 5–8; significant differences from the respective $S_2 - S_1$ controls: †$P<0.05$; ††$P<0.01$.

experiments with the frequency of 3 Hz. This becomes even more evident when the tritium overflowed evoked by one electrical pulse is calculated at 3 Hz or 0.3 Hz and the effects of clonidine or yohimbine are compared (Table 2).

At 0.3 Hz in the presence of 4β-PDB, the evoked overflow was about 4% of tissue tritium (Figures 3b and 4b; hatched horizontal bars) and similar to that obtained at a stimulation frequency of 3 Hz in the absence of 4β-PDB (Figures 3a and 4a: open horizontal bars). The inhibitory action of clonidine expressed as % of the respective S_1 value was decreased as compared to the controls obtained at 0.3 Hz in the absence of the phorbol ester (Figure 3a). However, in contrast to the experiments at 3 Hz, the inhibitory action of clonidine at 0.3 Hz was enhanced by 4β-PDB in absolute terms (Figure 3b: hatched vertical columns) and was similar to that obtained at a stimulation frequency of 3 Hz in the absence of 4β-PDB (Figure 3a: open vertical columns). The yohimbine-induced increase ($S_2 - S_1$) of the stimulation evoked overflow of tritium at 0.3 Hz was enhanced in the presence of 4β-PDB (Figure 4b: vertical columns). However, if the antagonistic effects was related to the respective S_1 an apparent attenuation was seen.

The effect of 4β-phorbol 12,13-dibutyrate following pretreatment with islet-activating protein or N-ethylmaleimide

Pretreatment of rabbit hippocampal slices with islet-activating protein (8 μg ml^{-1}; 18 h; Figure 5a: shaded horizontal bar) enhanced the electrically-evoked overflow of tritium by more than 100% as compared to the corresponding control value (18 h without IAP; Figure 5a: open horizontal bar). In the control slices, but not in the slices pretreated with IAP, S_2 was always enhanced.

The facilitatory effect of pretreatment with N-ethylmaleimide (30 μM; 30 min; Figure 5b: stippled

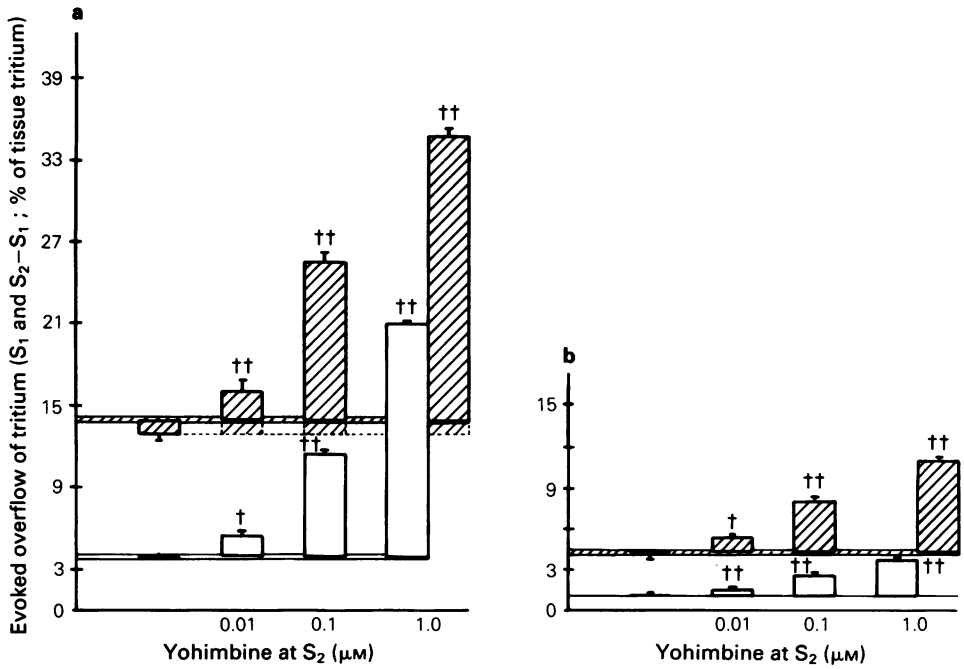

Figure 4 Effects of yohimbine on the evoked overflow of tritium in the absence (open columns/bars) or the presence (hatched columns/bars) of 4β-phorbol 12,13-dibutyrate (4β-PDB, 1 μM; throughout superfusion). Yohimbine was added to the superfusion medium from 15 min before S_2 onwards. Cocaine (30 μM) was present in all experiments throughout superfusion. The horizontal bars represent $S_1 \pm$ s.e.mean (expressed as % of tissue tritium) of all experiments. $S_2 - S_1$ (expressed as % of tissue tritium) are shown by vertical columns; vertical lines represent s.e.mean. The horizontal broken lines represent $S_2 - S_1$ of control. Stimulation was performed either at a stimulation frequency of 3 Hz (a) or of 0.3 Hz (b). Number of experiments of each group: 5–8; significant differences from the respective $S_2 - S_1$ controls: †$P < 0.05$; ††$P < 0.01$.

horizontal bar) on the evoked overflow of tritium was not so pronounced and amounted to an increase of about 70% as compared to the untreated slices (Figure 5b: open horizontal bar).

4β-PDB, applied 15 min before S_2, increased the evoked overflow of tritium in control slices incubated in medium for 30 min or 18 h, in the absence of N-ethylmaleimide or islet-activating protein, by com-

Table 2 Effects of clonidine and yohimbine on the evoked overflow of tritium per single pulse at 3 or 0.3 Hz

	Inhibition by clonidine		*Enhancement by yohimbine*	
S_1 (%)/pulse	*$S_2 - S_1$ (%)/pulse*	*% of S_1*	*$S_2 - S_1$ (%)/pulse*	*% of S_1*
3 Hz				
0.011	0.004	− 36	0.048	+ 436
0.3 Hz				
0.029	0.023	− 79	0.072	+ 248

[^{3}H]-noradrenaline labelled hippocampal slices were stimulated twice electrically (2 ms, 24 mA, 5 V cm^{-1}) at 3 Hz (= 360 pulses) or 0.3 Hz (= 36 pulses) for 2 min each. S_1 (as % of tissue tritium) was 3.93 ± 0.103 at 3 Hz (n = 36) and 1.04 ± 0.031 at 0.3 Hz (n = 38). Clonidine or yohimbine were administered from 15 min before S_2 onwards. Cocaine (30 μM) was present throughout superfusion. Effects of clonidine (0.03 μM) and yohimbine (1 μM)/pulse as % of tissue tritium or as % of the respective S_1 control value are given. Data shown are means of 5–8 observations.

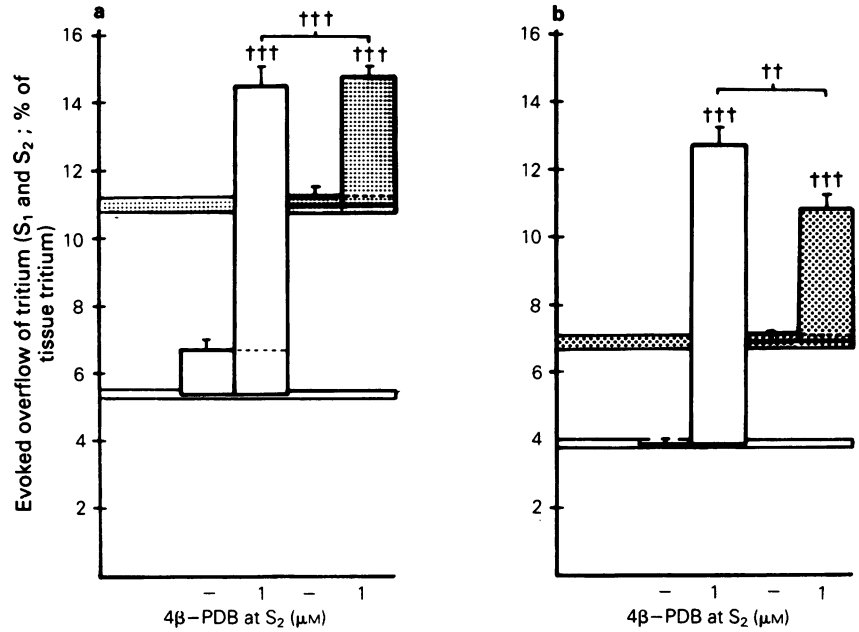

Figure 5 Effects of 4β-phorbol 12,13-dibutyrate (4β-PDB) on the electrically-evoked overflow of tritium from slices of the rabbit hippocampus pretreated with either (a) islet-activating protein or (b) N-ethylmaleimide (NEM). Pretreatment was performed either with islet-activating protein (8 μg ml^{-1}; shaded horizontal bars and vertical columns) in (a) for 18 h or with NEM (30 μM; stippled horizontal bars and vertical columns) in (b) for 30 min. Afterwards the slices were labelled with [^{3}H]-noradrenaline, superfused and stimulated electrically twice (3 Hz, 2 ms, 24 mA, 5 V cm^{-1}). 4β-PDB (1 μM) was added to the superfusion medium from 15 min before S_2 onwards. Cocaine (30 μM) was present in all experiments throughout superfusion. The horizontal bars represent S_1 ± s.e.mean (expressed as % of tissue tritium) of all experiments. $S_2 - S_1$ (expressed as % of tissue tritium) are shown by vertical columns; vertical lines represent s.e.mean. Broken horizontal lines represent $S_2 - S_1$ of control. Numbers of experiments of each group: 6–11; significant differences: ††$P < 0.01$; †††$P < 0.001$.

parable amounts (Figure 5a,b; open vertical columns). This phorbol ester-induced enhancement was strongly reduced following pretreatment of the slices in the presence of islet-activating protein (Figure 5a: shaded vertical columns) or N-ethylmaleimide (Figure 5b: stippled vertical columns).

Discussion

Involvement of protein kinase C in exocytotic release of noradrenaline

A crucial role of PKC in secretion processes has been demonstrated for a variety of cell types by the use of certain phorbol esters as activators of this enzyme (Nishizuka 1984). Recently, we reported that the electrically-evoked noradrenaline release in rabbit hippocampal slices was enhanced by TPA and 4β-PDB (Allgaier & Hertting, 1986; Algaier *et al.*, 1986b). The present paper extends these observations and attempts to characterize further the actions of phorbol esters.

Up to now all investigations with phorbol esters as activators of PKC point to the possibility of an involvement of PKC in controlling neurotransmitter release both in the central (Baraban *et al.*, 1985b; Allgaier & Hertting, 1986; Allgaier *et al.*, 1986b; Malenka *et al.*, 1986; Tanaka *et al.*, 1986; Feuerstein *et al.*, 1987) and in the peripheral (Wakade *et al.*, 1985; 1986; Baraban *et al.*, 1985a) nervous system. In the present paper, the conclusion that phorbol esters enhance noradrenaline release as a result of their effect on PKC is mainly supported by three observations discussed below; the facilitatory effects of two potent PKC activators, TPA and 4β-PDB (Castagna *et al.*, 1982), the lack of effects of phorbol compounds which do not activate PKC, and the inhibitory effect of the PKC inhibitor polymyxin B.

(1) The investigation of the concentration-dependence of the increase of noradrenaline release caused by TPA and by 4β-PDB showed that 4β-PDB is much more potent than TPA. The difference in the ability of TPA and 4β-PDB to enhance noradrenaline release in hippocampal slices, despite their similar potency to activate purified PKC *in vitro* (Castagna *et al.*, 1982), may be due to the higher lipid solubility of TPA as compared to 4β-PDB. It has previously been found in electrophysiological studies, that responses to lipid soluble drugs are extremely slow in brain slices (Dunwiddie & Fredholm, 1985). Possibly, lipophilic drugs need more time to equilibrate in the extracellular space as compared to more hydrophilic analogues (Dunwiddie & Fredholm, 1985).

(2) 4-*O*-methyl-TPA and the isomer of 4β-PDB, 4α-PDB, do not stimulate PKC (Gschwendt *et al.*, 1984; Ashendel, 1985). Since in the present investigation both compounds were without any effect on noradrenaline release (Figure 2), it may be concluded that the active phorbol esters, 4β-PDB and TPA, cause an enhancement of noradrenaline release by activating PKC. The additional observation that 4β-PDB increased the evoked noradrenaline release only in the presence of Ca^{2+} and the absence of tetrodotoxin (Table 1), indicates that this phorbol ester-induced activation of PKC may directly interfere with events regulating action potential-induced exocytotic release of noradrenaline.

(3) The conclusion that phorbol esters enhance transmitter release via activation of PKC is also supported by the finding that polymyxin B, a rather selective inhibitor of PKC (Kuo *et al.*, 1983), reduced the stimulation-evoked release of noradrenaline (Figure 1; and Allgaier & Hertting, 1986) and 5-hydroxytryptamine (Feuerstein *et al.*, 1987) from slices of the rabbit hippocampus and of acetylcholine from slices of the guinea-pig caudate nucleus (Tanaka *et al.*, 1986). Therefore a physiological involvement of PKC in the mechanism of stimulus-secretion-coupling can be assumed.

PKC involved in the modulation of noradrenaline release in rabbit hippocampal slices appears to be located presynaptically on the nerve terminals themselves or their immediate vicinity. Effects of the phorbol esters at the soma-dendritic area of the neurone and hence an influence on the firing rate of the neurone can be excluded, since in the *in vitro* hippocampal slice preparation noradrenergic nerve terminals are cut off from their cell bodies in the locus coeruleus. Also results from electrophysiological studies (Malenka *et al.*, 1986) and the finding that phorbol esters enhanced only the Ca^{2+}-dependent release of neurotransmitters (this paper and: Wakade *et al.*, 1985; Zurgil & Zisapel, 1985) point to an activation of presynaptically located PKC. Biochemical studies have demonstrated the presence of presynaptic PKC in brain slices (Girard *et al.*, 1985), its activation during depolarization (Wu *et al.*, 1982) and phosphorylation of membrane proteins from nerve terminals by PKC (Wu *et al.*, 1982).

Mechanism of the enhancement of noradrenaline release following protein kinase C activation

By blockade or functional impairment of the high affinity reuptake system for noradrenaline the evoked tritium overflow is strongly increased. However, that the phorbol ester-induced enhancement of noradrenaline release from rabbit hippocampal slices is caused by a direct or indirect action on the uptake system can be excluded from the present results, since all experiments were performed in the presence of the reuptake inhibitor cocaine (30 μM).

Noradrenergic nerve terminals of the rabbit hippocampus are endowed with inhibitory α_2-autoreceptors probably coupled to a regulatory N-protein (Allgaier *et al.*, 1985; 1986a). Therefore, the evoked noradrenaline release is strongly enhanced either by autoreceptor blockade with yohimbine (Jackisch *et al.*, 1984) or, as shown recently, by an inhibition of the feedback mechanism at the level of the N-protein by islet-activating protein or N-ethylmaleimide (Allgaier *et al.*, 1985; Allgaier *et al.*, 1986a). Hence, we investigated, whether the phorbol ester-induced PKC activation also impairs directly the α_2-autoreceptor-coupled feedback inhibition of noradrenaline release or acts by a different mechanism. Since changes in the concentration of the endogenous α_2-agonist noradrenaline have to be taken into account, when investigating the effects of exogenous agonists or antagonists in the presence of drugs or experimental conditions which strongly affect the concentration of the endogenous agonist (Reichenbacher *et al.*, 1982), the experiments were performed at two different stimulation frequencies. At a stimulation frequency of 3 Hz, in the absence of 4β-PDB, and at 0.3 Hz, in the presence of 4β-PDB, the evoked tritium overflow was comparable. However, it should be emphasized that similar S_1-values, obtained by stimulation under different conditions, are only an approximation to a similar autoinhibitory state, and do not reflect identical 'biophase' concentrations of endogenous noradrenaline, when the next pulse occurs.

The following results indicate, in contrast to the data obtained with islet-activating protein or N-ethylmaleimide mentioned above, that the enhancement of noradrenaline release caused by 4β-PDB does not seem to be due to effects on the α_2-autoinhibitory feedback system.

(1) At 3 Hz, in the absence of 4β-PDB, yohimbine caused a strong enhancement of the evoked noradrenaline release (Figure 4a; note that the increase is expressed as % of the tissue tritium content). The

effect of yohimbine was increased in the presence of 4β-PDB, reflecting the enhanced autoinhibition due to the higher concentration of endogenous noradrenaline in the synaptic cleft. Accordingly, in the latter situation the inhibitory effect of the α_2-agonist clonidine was diminished (Figure 3a).

At 0.3 Hz (without 4β-PDB) the effect of yohimbine, in comparison to that at 3 Hz, was lessened in consequence of the decreased biophase concentrations (Table 2). 4β-PDB, administered throughout superfusion, at 0.3 Hz increased the evoked noradrenaline release up to the level obtained at 3 Hz. Yet, the effect of yohimbine was reduced compared to that at 3 Hz without phorbol ester (in spite of comparable S_1 values). S_1 values are only an inaccurate measure for biophase concentrations, and the fact that the actual concentration of noradrenaline in the vicinity of the autoreceptor at the time of the occurrence of the subsequent pulse has already declined, due to the ten fold spacing of the pulses, has to be taken into account.

Since the phorbol ester *per se* caused a strong increase of noradrenaline release by more than 2 fold, it is important to regard the yohimbine-dependent changes in the evoked neurotransmitter release in absolute terms and not its relative effects. Please note, that both at 3 and 0.3 Hz the effects of the phorbol ester and yohimbine were additive or even more than additive. This finding is inconsistent with the idea that both drugs interact directly at a common mechanism. Therefore, α_2-adrenoceptor blockade by the phorbol ester, or a post-receptor inhibition of the autoinhibitory feedback mechanism, seems not to be the reason for the phorbol ester-induced enhancement of evoked noradrenaline release.

(2) The inhibitory effect of the α_2-adrenoceptor agonist clonidine was significantly reduced in the presence of 4β-PDB at a stimulation frequency of 3 Hz. At first consideration, this finding might be interpreted as an impairment of the α_2-autoreceptor mechanism by PDB. However, a detailed analysis of the effects of clonidine at two different stimulation frequencies shows that its reduced effectiveness in the presence of PDB may be better explained by an enhancement of the concentration of noradrenaline in the synaptic cleft (biophase) following 4β-PDB. This becomes especially evident when comparing the effects of clonidine at 0.3 Hz in the presence of 4β-PDB (Figure 3b), with those at 3 Hz in the absence of 4β-PDB (Figure 3a). Under these conditions the inhibitory effects of clonidine in the absence (but at 3 Hz) and presence (but at 0.3 Hz) of 4β-PDB were quite similar.

(3) Although, as outlined above, the α_2-autoreceptor-coupled mechanism seems not to be affected by phorbol ester-induced PKC stimulation, inversely the 4β-PDB-induced increase in noradrenaline release was significantly reduced following impairment of the α_2-autoreceptor mechanism with islet-activating protein or N-ethylmaleimide. Hence, at first sight the PKC-mediated stimulation of noradrenaline release appears to depend on steps which are influenced by the α_2-autoreceptor mechanism. However, under another condition of reduced feedback inhibition by endogenous noradrenaline (stimulation at 0.3 Hz), the PDB effect was enhanced rather than reduced (compare the S_1-values in Figure 3, horizontal bars). Therefore, we assume that the reduced effects of 4β-PDB following pretreatment with islet-activating protein or N-ethylmaleimide are unrelated to the impairment of the autoinhibitory feedback mechanism. However, since the 4β-PDB effect was reduced under these conditions, it may be speculated that another regulatory N-protein is involved in steps following PKC activation by phorbol esters.

The mechanisms underlying the phorbol ester-induced enhancement of neurotransmitter release are still under discussion. A blockade of potassium channels and hence a prolongation of the action potential in the terminal may occur and would increase transmitter release: the presence of several potassium channels in isolated nerve terminals of rat brain (Bartschat & Blaustein, 1985) and the blockade of similar potassium channels in hippocampal pyramidal cells by phorbol esters (Baraban *et al.*, 1985) has been shown recently. Alternatively phorbol esters may regulate the activity of Ca^{2+}-channels (De Riemer *et al.*, 1985; Wakade *et al.*, 1986). However, observations about phorbol ester effects on Ca^{2+}-channels have, as yet, been contradictory: in *Aplysia* neurones phorbol esters directly augmented Ca^{2+}-currents (De Riemer *et al.*, 1985), whereas the enhancement of dopamine release from brain neurones was independent of extracellular Ca^{2+}-concentrations and appeared to be due to increases in the affinity of Ca^{2+} to PKC (Zurgil & Zisapel, 1985). Recently, even an inhibitory control of voltage-gated Ca^{2+}-channels exerted by phorbol esters has been shown (Di Virgilio *et al.*, 1986; Messing *et al.*, 1986).

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