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Protein kinase C and presynaptic modulation of acetylcholine release in rabbit hippocampus

[1]Clemens Allgaier, Beate Daschmann, Hua Yu Huang & Georg Hertting

Institute of Pharmacology, Hermann-Herder-Straße, 5 D-78 Freiburg i. Br., Federal Republic of Germany

1 The involvement of protein kinase C in the presynaptic modulation of stimulated acetylcholine release was investigated in rabbit hippocampus.

2 Slices of the rabbit hippocampus, labelled with [^{3}H]-acetylcholine, were superfused with medium and stimulated electrically during superfusion.

3 The protein kinase C activating phorbol ester 4β-phorbol 12,13-dibutyrate (4β-PDB) enhanced the electrically evoked tritium overflow in a concentration-dependent manner. Its biologically inactive 4α-isomer was without any effect on transmitter release.

4 The protein kinase C inhibitor polymyxin B decreased the stimulation-evoked tritium overflow and counteracted the enhancement of release caused by 4β-PDB.

5 The stimulation-evoked tritium overflow was facilitated when the muscarine receptor antagonist atropine was present. The effects of both atropine and 4β-PDB, given in combination, were additive.

6 The net inhibition of the evoked tritium overflow caused by the muscarine receptor agonists carbachol and oxotremorine was similar, irrespective of whether 4β-PDB was present or not.

7 Similar results to those for muscarine autoreceptor-mediated inhibition, were obtained for inhibition of the stimulated tritium overflow caused by the adenosine receptor agonist (−)-N^6-(R-phenylisopropyl)-adenosine ((−)-PIA) and the opioid receptor agonist ethylketocyclazocine (EKC). The net inhibition of both agonists was independent of the presence of the phorbol ester.

8 The above results provide further evidence for participation of a presynaptically located protein kinase C in the modulation of acetylcholine release. However, the modulatory mechanisms which are coupled to presynaptic receptors and mediate inhibition of release seem not to be directly affected by protein kinase C.

Introduction

The role of inositol triphosphate and diacylglycerol as second messengers has been well established during the last few years (Berridge, 1984; Abdel-Latif, 1986). Both inositol triphosphate and diacylglycerol are formed by enzymatic cleavage of the membrane compound phophatidylinositol 4,5-biphosphate. After hydrolysation of the phospholipid, inositol triphosphate is released into the cytosol for mobilizing Ca^{2+} from intracellular stores, whereas diacylglycerol is operating within the plane of the membrane for activating protein kinase C. The action of endogenous diacylglycerol is mimicked by tumour-promoting phorbol esters. Results obtained from experiments with phorbol esters as activators of protein kinase C or enzyme inhibitors like polymyxin B (Kuo *et al.*, 1983) indicate a possible participation of protein kinase C in the modulation of neurotransmitter release from noradrenergic (Allgaier & Hertting, 1986; Allgaier *et al.*, 1986; 1987) and 5-hydroxytryptaminergic (Feuerstein *et al.*, 1987) nerve terminals in the rabbit hippocampus.

In the present study, the possible involvement of protein kinase C in the modulation of the stimulation-evoked [^{3}H]-acetylcholine release from rabbit hippocampal slices has been investigated. As cholinergic nerve terminals of the rabbit hippocampus are endowed with muscarine autoreceptors which inhibit the electrically-evoked release of acetylcholine (Strittmatter *et al.*, 1982), experiments were performed to see whether the effects of the phorbol ester were, at least in part, due to an inactivation of the autoinhibitory feedback mechanism.

In addition to the muscarine autoreceptor mechanism, evoked release of acetylcholine from rabbit

[1] Author for correspondence.

hippocampus is inhibited by activation of presynaptically located A_1-adenosine (Jackisch *et al.*, 1984) and κ-opioid receptors (Jackisch *et al.*, 1986). Therefore, further work to discover whether these heteroreceptor-coupled mechanisms are affected by phorbol esters was undertaken.

Methods

Superfusion experiments

Superfusion experiments have been performed as reported previously by Strittmatter *et al.* (1982). In brief: slices (400 μm; 4–7 mg) were 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 5% CO_2 in O_2, pH 7.4) containing 0.1 μmol l^{-1} [^{3}H]-choline (80 Ci $mmol^{-1}$) for 30 min at 37°C. Subsequently, the ^{3}H-labelled slices were superfused with medium containing hemicholinium-3 (10 μM) for 140 min. Other drugs were present throughout superfusion in addition to hemicholinium-3 as indicated in the text. Collection of 5 min samples of the superfusate was started after 45 min. After 60 (S_1) and 120 min (S_2) of superfusion, electrical stimulation was performed with vertical rectangular pulses (3 Hz, 2 ms, 24 mA, 5 V cm^{-1}) for 2 min each. Drugs were added between S_1 and S_2 to the superfusion medium in order to test their effects on acetylcholine release. At the end of superfusion, the slices were removed from the chambers and solubilized in Soluene 350 (Packard Instrument, Frankfurt, FRG). Tritium determination of the superfusates and the solubilized tissue was made by liquid scintillation counting.

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). 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 15–20 min after the onset of stimulation. The evoked-overflow of tritium (S_1, S_2) was expressed as a percentage fraction of the tritium content of the slice at the start of the respective stimulation period. As shown by Richardson & Szerb (1974) electrical stimulation of brain slices preincubated with [^{3}H]-choline causes the release of only [^{3}H]-acetylcholine. Therefore, the evoked overflow of tritium represents a good measure of the release of [^{3}H]-acetylcholine. Drug effects on acetylcholine release were evaluated by calculating the ratios S_2/S_1 of the overflow evoked by the two stimulation periods. Effects of drugs on basal tritium outflow are shown by the ratio b_2/b_1 of the fractional rate immediately before S_2 (115–120 min) and the fractional rate immediately before S_1 (55–60 min).

Statistics

All results are shown as arithmetic means ± s.e.mean. Differences in the means of the S_2/S_1 ratios of treated groups and their corresponding controls were tested with ANOVA. Bartlett's test, with a minimum level of significance of 10%, was applied to refute the null hypothesis (homogeneity of variances, goodness of fit for normal distribution). The significance of differences between the treated groups was determined by Student's *t* test (two-tailed) using the error mean square of ANOVA as an estimate of the standard deviation of all means.

Drugs

The following drugs were used: [methyl-^{3}H]-choline chloride (80 Ci $mmol^{-1}$; Amersham Buchler, Braunschweig, FRG); (−)-N^6-(R-phenylisopropyl)-adenosine ((−)-PIA; Boehringer, Mannheim, FRG); atropine sulphate and carbachol HCl (Merck, Darmstadt, FRG); polymyxin B sulphate (Serva, Heidelberg, FRG), 4β-phorbol 12,13-dibutyrate (4β-PDB), hemicholinium-3, oxotremorine sesquifumarate, physostigmine salicylate and tetrodotoxin (Sigma, München, FRG), 4α-PDB (Paesel, Frankfurt, FRG). Ethylketocyclazocine methanesulphonate (EKC) was obtained from Dr A.E. Soria (Sterling-Winthrop, Rensselear. N.Y., U.S.A. Stock solutions (10 mM) of the phorbol esters were made in dimethylsulphoxide. The maximum concentration of dimethylsulphoxide used (0.1%) was without any effect on basal or on stimulation-evoked tritium outflow.

Results

Effects of phorbol esters

Rabbit hippocampal slices, preincubated in the presence of the [^{3}H]-acetylcholine precursor, [^{3}H]-choline, were superfused in general with medium containing the choline uptake inhibitor hemicholinium-3 (10 μM). During superfusion the tissue was stimulated twice electrically. The tumour-promoting phorbol ester 4β-PDB, a potent activator of protein kinase C (Castagna *et al.*, 1982), increased the evoked tritium overflow in a concentration-dependent manner (Figure 1). 4β-PDB 0.1 μM caused a 14% increase, 1 μM of the phorbol ester produced a 48% and 10 μM a 72% increase. At the latter two concentrations basal tritium outflow was also increased (Table 1). Similar effects of 4β-PDB were seen when superfusion was

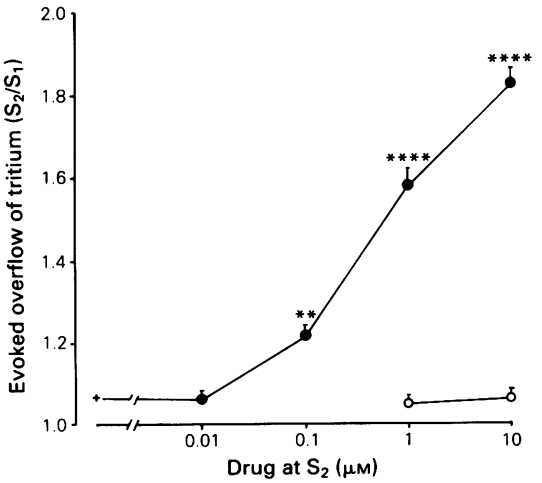

Figure 1 Effects of phorbol esters on the electrically-evoked tritium overflow from rabbit hippocampus. Slices, preincubated with medium containing [^{3}H]-choline, were superfused in the presence of hemicholinium-3 (10 μM) and stimulated twice electrically (S_1; S_2) during superfusion. 4β-Phorbol 12, 13-dibutyrate (4β-PDB, ●) or its isomer 4α-PDB (○) were present in the superfusion medium from 15 min before S_2 onwards. Effects of the phorbol esters on the stimulation-evoked overflow of tritium are expressed by the ratio S_2/S_1 of the overflow evoked by the two stimulation periods. Means of S_2/S_1 ± s.e.mean (shown by vertical bars) are given. S_1 ± s.e.mean (in % of tissue tritium content at the onset of stimulation) of all experiments was 3.41 ± 0.14% (n = 38). Numbers of experiments per group: 5–7; significant differences from drug-free control (+): $^{**}P<0.01$; $^{***}P<0.0001$.

performed without hemicholinium-3 (S_2/S_1 = 1.02 ± 0.04, n = 5 for controls; S_2/S_1 = 1.60 ± 0.04, n = 5, for 4β-PDB 1 μM).

In contrast to 4β-PDB, its stereoisomer 4α-PDB, which is biologically inactive, was without any effect both on evoked (Figure 1) or basal (not shown) tritium outflow up to a concentration of 10 μM.

In order to exclude the possibility that the phorbol ester-induced enhancement of the evoked tritium overflow was, at least in part, independent of Ca^{2+} and insensitive to tetrodotoxin (TTX), slices were superfused from 15 min before S_2 onwards, either with Ca^{2+}-free or TTX (0.3 μM)-containing medium. Under these conditions, tritium overflow evoked by electrical field stimulation was almost totally blocked, even in the presence of 4β-PDB (1 μM; Table 2).

The effect of 4β-PDB was studied in the presence of different concentrations of extracellular Ca^{2+} (0.65–5.2 mM). The Ca^{2+}-concentration of the medium was changed from 1.3 mM to the indicated levels from 15 min before the second stimulation onwards (Figure 3). At each Ca^{2+}-concentration the evoked overflow of tritium was increased by the phorbol ester.

Effects of polymyxin B

Since 4β-PDB, as an activator of protein kinase C, enhanced the stimulation-evoked overflow of tritium, the effect of polymyxin B as an inhibitor of this enzyme (Kuo *et al.*, 1983) was studied. Polymyxin B was administered from 15 to 40 min before S_2 onwards, in concentrations up to 100 μM. Polymyxin B diminished the evoked tritium overflow in a time- and concentration-dependent manner (Table 3). Basal outflow was increased by 100 μM polymyxin B after a superfusion period of 15 min by 13% (b_2/b_1 = 0.74 ± 0.03, n = 4; $P<0.05$ vs. control see Table 1), and after 40 min of superfusion by 42% (b_2/b_1 = 0.95 ± 0.04, n = 9;

Table 1 Effect of 4β-phorbol 12, 13-dibutyrate (4β-PDB) on basal outflow of tritium from hippocampal slices preincubated in the presence of [^{3}H]-choline

Drug present at S_2 (μM)	b_2/b_1	*Increase* (% of control)	n
—	0.67 ± 0.02	—	7
4β-PDB 0.01	0.68 ± 0.02	1	7
4β-PDB 0.10	0.69 ± 0.02	3	6
4β-PDB 1.00	0.86 ± 0.02***	28	5
4β-PDB 10.00	0.92 ± 0.02****	37	5

After preincubation, the slices were superfused with medium containing hemicholinium-3 (10 μM) and stimulated twice electrically (S_1; S_2). 4β-PDB was present from 15 min before S_2 onwards in the concentrations indicated. Drug effects on basal outflow are expressed as ratio b_2/b_1 between the fractional rate of outflow immediately before S_2 (95–100 min) and the fractional rate of outflow immediately before S_1 (55–60 min). Means ± s.e.mean of n observations are given; significant differences from drug-free control: $^{***}P<0.001$; $^{****}P<0.0001$.

Table 2 4β-Phorbol 12, 13-dibutyrate (4β-PDB) effect on the electrically-evoked overflow of tritium 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	b_2/b_1	n
—	+	—	1.08 ± 0.03	0.67 ± 0.03	7
+	+	—	1.57 ± 0.03	0.86 ± 0.03	8
—	—	—	0.06 ± 0.03	0.81 ± 0.03	7
+	—	—	0.11 ± 0.03	1.09 ± 0.03	7
—	+	+	0.03 ± 0.04	0.61 ± 0.04	4
+	+	+	0.08 ± 0.04	0.79 ± 0.04	4

^{3}H-labelled slices were superfused in the presence of hemicholinium-3 (10 μM) and stimulated electrically twice (S_1: S_2). 4β-PDB was administered from 15 min before S_2 onwards in the presence or absence of Ca^{2+} (Ca^{2+} removal from 15 min before S_2 onwards) and in the presence of TTX (present from 15 min prior to S_2). Means of S_2/S_1 ± s.e.mean of *n* observations are given. Basal outflow (b_2/b_1) is expressed as ratio between the fractional rate of outflow immediately before S_2 (115–120 min) and the fractional rate of outflow immediately before S_1 (55–60 min).

$P < 0.0001$). Lower concentrations of polymyxin B used were without effect on basal outflow.

Influence of polymyxin B on the action of 4β-PDB

To discover whether the enhancement of the evoked tritium overflow caused by 4β-PDB is diminished by polymyxin B, the effect of the phorbol ester (1 μM) was determined in the presence of the protein kinase inhibitor (100 μM). Both drugs were added to the superfusion medium between S_1 and S_2 (polymyxin B 25 min before the addition of 4β-PDB).

The net effect of the phorbol ester (the difference between the control value and the value obtained for 4β-PDB) was 0.50 ± 0.04 (Table 4). It was reduced to 0.32 ± 0.04 in presence of polymyxin B (the difference between the value obtained for polymyxin B alone and for polymyxin B plus 4β-PDB; Table 4). Accordingly, the net effect of polymyxin B was increased. However, it should be noted that the relative inhibitory effect of the putative protein kinase C inhibitor remained unchanged.

Basal outflow was strongly enhanced, when both drugs were given in combination (Table 4).

Phorbol ester effect and muscarine autoreceptor mechanism

The stimulation-evoked acetylcholine release in rabbit hippocampus is slightly augmented by the muscarine receptor antagonist, atropine (Strittmater *et al.*, 1982). This result suggests inhibition of release caused by endogenous acetylcholine. In the experiments presented here, atropine (2 μM) administered in the superfusion medium before the second stimulation period, enhanced the electrically-evoked overflow of tritium by more than 30% (Figure 2). In order to exclude the possibility that the phorbol ester-induced enhancement of the evoked tritium overflow is due, at least in part, to an impairment of the autoinhibitory feedback mechanism, experiments were performed where atropine and 4β-PDB were present in medium

Table 3 (a) Time- and (b) concentration-dependence of the effect of polymyxin B on the electrically-evoked tritium overflow from rabbit hippocampal slices preincubated in the presence of [^{3}H]-choline

Polymyxin B at S_2 (μM)	S_2/S_1	*Inhibition* (% of control)	n
a			
—	1.09 ± 0.02	—	7
100; 15 min before S_2	0.74 ± 0.03***	32	4
100; 25 min before S_2	0.61 ± 0.02****	44	7
100; 40 min before S_2	0.45 ± 0.02****	58	9
b			
—	1.09 ± 0.01	—	7
10	1.03 ± 0.02*	6	5
33	0.95 ± 0.02***	13	6
100	0.61 ± 0.02****	44	7

Slices superfused with medium containing hemicholinium-3 (10 μM) were stimulated twice electrically (S_1; S_2). (a) Polymyxin B (100 μM) was administered from 15, 25 or 40 min before S_2 onwards. (b) Polymyxin B was added in the indicated concentrations from 25 min before S_2 onwards. Means of S_2/S_1 ± s.e.mean are given. S_1 ± s.e.mean (in % of tissue tritium at the onset of stimulation) was 3.17 ± 0.10% ($n = 25$). Significant differences from drug-free control: *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

Table 4 Effect of 4β-phorbol 12, 13-dibutyrate (4β-PDB) on the stimulation-evoked overflow of tritium from [^{3}H]-choline-preincubated brain slices in the presence of polymyxin B

Drugs at S_2 (μM)	S_2/S_1	*Net effect of 4β-PDB*	b_2/b_1	n
—	1.03 ± 0.03	—	0.64 ± 0.03	5
4β-PDB 1	1.53 ± 0.03****	0.50 ± 0.04	0.79 ± 0.03**	5
Polymyxin B 100	0.45 ± 0.03****	—	1.04 ± 0.04****	4
Polymyxin B 100 + 4β-PDB 1	0.77 ± 0.03****	0.32 ± 0.04	1.28 ± 0.03****	6

After ^{3}H-labelling, the slices were superfused and stimulated twice (S_1; S_2). Polymyxin B was administered from 40 min, 4β-PDB from 15 min before S_2 onwards. S_1 ± s.e.mean (expressed as % of tissue tritium at the onset of stimulation) for all experiments was 2.96 ± 0.06% (n = 20). The ratios S_2/S_1 of the overflow evoked by the two stimulation periods of each group are given. Effects on basal outflow are expressed as ratio b_2/b_1 between the fractional rate of outflow immediately before S_2 (115–120 min) and the fractional rate of outflow immediately before S_1 (55–60 min). Significant differences from drug-free control: **$P<0.01$; ****$P<0.0001$.

alone or in combination. The increase of the tritium overflow caused by both drugs was slightly more than an additive affect and amounted to 112%, compared to 34% by atropine and to 62% by 4β-PDB, administered alone (Figure 2).

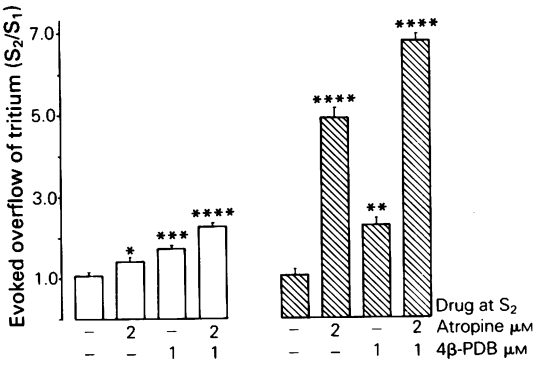

Figure 2 Effects of atropine and 4β-phorbol 12, 13-dibutyrate (4β-PDB), administered alone or in combination, on evoked tritium overflow from [^{3}H]-acetylcholine prelabelled brain slices. During superfusion, performed either in the presence of hemicholinium-3 (10 μM; open columns) or hemicholinium-3 (10 μM) and physostigmine (1 μM; hatched columns) throughout superfusion, the slices were stimulated twice electrically (S_1; S_2). Atropine and/or 4β-PDB were present from 15 min before S_2 onwards. Means of S_2/S_1 ± s.e.mean (shown by vertical bars) are given. S_1 ± s.e.mean (in % of tissue tritium content at the onset of stimulation) in the presence of hemicholinium-3 was 2.54 ± 0.18% (n = 21); S_1 ± s.e.mean in the presence of hemicholinium-3 and physostigmine was 0.92 ± 0.02% (n = 24). Numbers of experiments per group: 5–7; significant differences from respective control: *$P<0.05$; **$P<0.01$; ***$P<0.0001$.

In the presence of the acetylcholine esterase inhibitor physostigmine, the inhibitory effect of endogenous acetylcholine was strongly augmented. The electrically-evoked overflow of tritium was reduced from about 3% of the tissue tritium content at

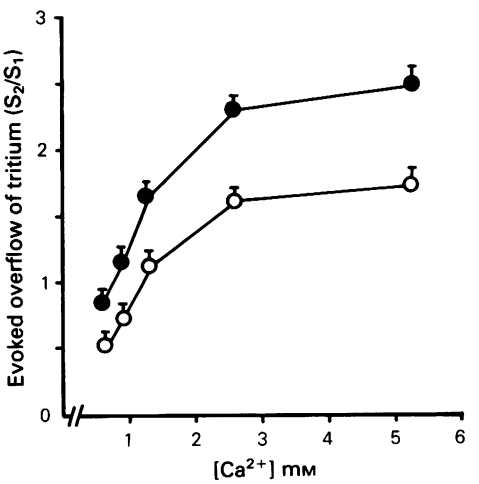

Figure 3 Rabbit hippocampal slices, preincubated in the presence of [^{3}H]-choline, were superfused with medium containing hemicholinium-3 (10 μM). During superfusion the ^{3}H-labelled slices were stimulated electrically twice (S_1; S_2). 4β-Phorbol 12,13-dibutyrate (4β-PDB) was administered from 15 min before S_2 onwards in the presence of various concentrations of Ca^{2+}. The Ca^{2+}-concentration of the medium was changed from 1.3 mM to the indicated concentrations 15 min before S_2 onwards. S_1 ± s.e.mean (in % of the tissue tritium content at the onset of stimulation) was 2.82 ± 0.07 (n = 67). Means of S_2/S_1 ± s.e.mean of each group and the respective 4β-PDB-induced increases of the evoked tritium overflow are given. Number of observations per group: 4–8.

the onset of stimulation, when hemicholinium-3 was present alone, to about 1%, when physostigmine was given in addition. The effects of atropine (2 μM) and 4β-PDB (1 μM), administered alone or in combination, were tested in the presence of physostigmine. The relative effects of both atropine and 4β-PDB on the evoked overflow of tritium were increased following inhibition of acetylcholine esterase up to 353% in the case of the antagonist and to 129% in the case of the phorbol ester, given alone, respectively. In the presence of physostigmine the effects of the phorbol ester and atropine were again slightly greater than additive. When both drugs were given in combination, the relative increase of the evoked overflow of tritium was to 528%. However, it should be emphasized, that the absolute effect of 4β-PDB (in terms of tissue tritium content), in contrast to that of atropine, was not increased by inhibition of the enzymatic cleavage of endogenous acetylcholine.

The electrically-evoked release of acetylcholine from brain slices was diminished by the muscarine receptor agonists carbachol (1 μM) by 35% and by oxotremorine (1 μM) by 67% (Table 5). Both carbachol and oxotremorine also showed an inhibitory effect on the evoked tritium overflow, when they were added to the medium in combination with 4β-PDB (Table 5). Since the overflow of tritium was increased in the presence of the phorbol ester, the relative inhibitory effects of both muscarine receptor agonists were reduced. However, the net effect of inhibition of the tritium overflow by carbachol and oxotremorine did not differ whether in the absence or presence of the phorbol ester (Table 5). The net effects were obtained by calculating the difference between the control value and the value obtained in the presence of the autoreceptor agonists and the difference between the value obtained for the phorbol ester alone and for the phorbol ester plus the respective agonist.

Adenosine and opioid receptor-mediated release modulation in the presence of 4β-PDB

The electrically-evoked tritium overflow from rabbit hippocampal slices is inhibited by activation of A_1-adenosine (Jackisch *et al.*, 1984) and κ-opioid-receptors (Jackisch *et al.*, 1986) which are located at the cholinergic nerve terminals. The effects of the A_1-adenosine receptor agonist (−)-PIA and the preferential κ-opioid receptor agonist EKC on the evoked tritium overflow were studied in the presence of 4β-PDB (Tables 6, 7) All drugs were given from 15 min before the second stimulation period onwards. As with the muscarine receptor agonists, the relative inhibitory effects of both heteroreceptor agonists were reduced in the presence of the phorbol ester, whereas their net effects (calculated by subtracting the respective S_2/S_1 values) remained unchanged compared to the results obtained in the absence of 4β-PDB (Table 6, 7). In contrast, the 4β-PDB-induced net enhancement of the evoked tritium overflow was not influenced under these conditions by activation of either the A_1- or the κ-opioid-heteroreceptor-coupled mechanisms.

Similar results were obtained when the experiments were performed during muscarine autoreceptor blockade in the presence of atropine (Tables 6, 7). Due to endogenous acetylcholine the evoked overflow of tritium was increased in the presence of the muscarine receptor antagonist. Therefore, under these conditions the net effect of inhibition of the evoked overflow by (−)-PIA and EKC was also enhanced. No significant difference between the net inhibitory effects caused by the heteroreceptor agonists in the presence or absence

Table 5 Effects of the muscarine receptor agonists carbachol and oxotremorine on the evoked tritium overflow in the presence of 4β-phorbol 12, 13-dibutyrate (4β-PDB)

Drug at S_2 (μM)	*S_2/S_1*	*Net effect of inhibition*	n
—	1.03 ± 0.02	—	8
Carbachol 1.0	0.67 ± 0.02****[a]	0.36 ± 0.03	6
Oxotremorine 1.0	0.34 ± 0.03****[a]	0.67 ± 0.04	4
4β-PDB 1.0	1.63 ± 0.02	—	8
Carbachol 1.0 + 4β-PDB 1.0	1.31 ± 0.03****[b]	0.32 ± 0.04	4
Oxotremorine 1.0 + 4β-PDB 1.0	0.98 ± 0.03****[b]	0.65 ± 0.04	4

Hippocampal slices incubated with [^{3}H]-choline were stimulated electrically twice (S_1; S_2) during superfusion. Hemicholinium-3 (10 μM) was present throughout. Superfusion with medium containing carbachol (1 μM) or oxotremorine (1 μM), alone, respectively, or in combination with 4β-PDB was started from 15 min before S_2 onwards. S_1 ± s.e.mean (in % of tissue tritium at the onset of stimulation) was 2.82 ± 0.08% (n = 34). n number of experiments. Significant inhibitory effects of the muscarine agonists on the evoked tritium overflow [a]compared to drug-free control, [b]compared to the value obtained in the presence of 4β-PDB: **$P<0.01$; ****$P<0.0001$.

Table 6 Effects of the adenosine receptor agonist (−)-N⁶-(R-phenylisopropyl)-adenosine ((−)-PIA) on the stimulated tritium overflow in the presence of 4β-phorbol 12, 13-dibutyrate (4β-PDB)

Drugs at S_2 (μM)					
(−)-PIA	*4β-PDB*	*Atropine*	*S_2/S_1*	*Net effect of inhibition*	n
a					
—	—	—	1.07 ± 0.04	—	7
1.0	—	—	0.74 ± 0.04****	0.33 ± 0.06	5
—	1.0	—	1.62 ± 0.03	—	7
0.1	1.0	—	1.33 ± 0.03****	0.29 ± 0.04	9
b					
—	—	2.0	1.45 ± 0.04	—	7
0.1	—	2.0	0.91 ± 0.04****	0.54 ± 0.06	9
—	1.0	2.0	2.20 ± 0.04	—	6
0.1	1.0	2.0	1.67 ± 0.03****	0.53 ± 0.05	10

Brain slices preincubated with [³H]-choline were superfused and stimulated twice electrically (S_1; S_2). Hemicholinium-3 (10 μM) was present throughout in all experiments. (a) (−)-PIA (0.1 μM) and/or 4β-PDB (1 μM), and (b) in addition, atropine (2 μM) were added to the superfusion medium from 15 min before S_2 onwards. S_1 ± s.e.mean (in % of the tissue tritium content at the onset of stimulation) was 2.85 ± 0.61 ($n = 60$). The ratios S_2/S_1 of the overflow evoked by the two stimulation periods of each group are given. Significance of the inhibitory effect of (−)-PIA in the absence or presence of 4β-PDB: ****$P < 0.0001$. n = number of experiments.

of the phorbol ester was seen.

Discussion

The involvement of protein kinase C in the modulation of acetylcholine release in rabbit hippocampus is supported by several findings. First, the protein kinase C activating phorbol ester 4β-PDB enhanced the electrically-evoked release of acetylcholine in a concentration-dependent manner (Figure 1). Second, its inactive 4α-isomer (Ashendal 1985), was without any effect up to a concentration of 10 μM (Figure 1). Third, polymyxin B, described by Kuo *et al.* (1983), as a rather selective protein kinase C inhibitor, reduced the stimulation-evoked transmitter release and, in addi-

Table 7 Effects of the opioid receptor agonist ethyketocyclazocine (EKC) on the stimulated tritium overflow in the presence of 4β-phorbol 12, 13-dibutyrate (4β-PDB)

Drugs at S_2 (μM)					
EKC	*4β-PDB*	*Atropine*	*S_2/S_1*	*Net effect of inhibition*	n
a					
—	—	—	1.05 ± 0.03	—	7
0.01	—	—	0.71 ± 0.03****	0.34 ± 0.04	9
—	1.0	—	1.62 ± 0.03	—	7
0.01	1.0	—	1.27 ± 0.03****	0.35 ± 0.04	7
b					
—	—	2.0	1.43 ± 0.05	—	7
0.01	—	2.0	1.00 ± 0.04****	0.43 ± 0.06	9
—	1.0	2.0	2.18 ± 0.05	—	6
0.01	1.0	2.0	1.66 ± 0.04****	0.52 ± 0.06	6

Brain slices incubated with [³H]-choline were superfused and stimulated twice electrically (S_1; S_2). Hemicholinium-3 (10 μM) was present throughout in all experiments. (a) EKC (0.01 μM), 4β-PDB (1 μM) and (b) in addition, atropine (2 μM) were added to the superfusion medium from 15 min before S_2 onwards, as indicated. S_1 ± s.e.mean was 2.82 ± 0.10 ($n = 58$). Significance of the inhibitory effect of (−)-PIA in the absence or presence of 4β-PDB: ****$P < 0.0001$. n = number of experiments.

tion, counteracted the enhancement of release caused by 4β-PDB (Table 4). As during preparation of the tissue slices the cholinergic nerve endings of the hippocampus are cut off from the neuronal cell bodies located in the septum, protein kinase C involved in release modulation by the phorbol ester appears to be located at or near to the synaptic site of the cholinergic nerve terminal.

The above results are in accordance both with biochemical studies where [^{3}H]-acetylcholine release from brain slices (Tanaka *et al.*, 1986; Versteeg & Florijn, 1987) and synaptosomes (Nichols *et al.*, 1987) has been measured and with electrophysiological studies performed on nerve muscle preparations (Haimann *et al.*, 1987; Murphy & Smith, 1987; Shapira *et al.*, 1987). During recent years the presence of a presynaptically located protein kinase C in brain slices (Girard *et al.*, 1985), and the phosphorylation of several membrane proteins, including a 87 kDa substrate, by protein kinase C during depolarization has been demonstrated (Wu *et al.*, 1982). As reported recently by Nichols *et al.* (1987), phosphorylation of the 87 kDa substrate by phorbol ester-activated protein kinase C in synaptosomes was in parallel with the enhancement of stimulated release. In contrast, the inactive 4α-phorbol esters were without effect either on release or phosphorylation.

The precise biochemical mechanism underlying the enhancement of release caused by phorbol esters is as yet not clear. The possibility that facilitation of release by 4β-PDB is due to a blockade of the high-affinity uptake mechanism for choline can be excluded, since superfusion was performed in the presence of the uptake inhibitor hemicholinium-3. The observation that the active phorbol ester predominantly potentiates Ca^{2+}-dependent and TTX-sensitive stimulation-evoked release (Table 2; basal outflow was only slightly increased, Table 1) points to a direct interference of phorbol ester-activated protein kinase C with exocytotic release mechanism. In addition, enhancement of evoked transmitter release by 4β-PDB cannot be caused by liberation of Ca^{2+} from intracellular stores.

As reported by Zurgil & Zisapel (1985), the enhancement of dopamine release from brain neurones caused by phorbol esters was higher at lower extracellular Ca^{2+} concentrations. This result is compatible with the idea of an increase of either the influx of Ca^{2+} or the affinity of Ca^{2+} for protein kinase C. However, these findings are in contrast to an electrophysiological study (Murphy & Smith, 1987), where the phorbol ester-induced increase of release remained unchanged in Ca^{2+}-deficient solutions and to experiments performed on PC12 cells, where voltage-dependent Ca^{2+} channels appeared to be under an inhibitory control (Di Virgilio *et al.*, 1986; Messing *et al.*, 1986). In our experiments the effect of 4β-PDB on evoked acetylcholine release was tested at various Ca^{2+}-concentrations from 0.65 mM to 5.2 mM (Figure 3). When Ca^{2+} was used below 0.65 mM, the stimulated release was almost abolished. The net increase caused by the phorbol ester was reduced at lower Ca^{2+}-concentrations (Figure 3). No clear and significant correlation was seen between extracellular Ca^{2+}-levels and the relative facilitatory effect of the phorbol ester.

Polymyxin B has been used by several groups investigating the mechanism of release of noradrenaline, 5-hydroxytryptamine and acetylcholine as a putative inhibitor of protein kinase C (Allgaier & Hertting, 1986; Wakade *et al.*, 1986; Tanaka *et al.*, 1986; Feuerstein *et al.*, 1987; Versteeg & Ulenkate, 1987). In each of these studies polymyxin B diminished the stimulated release and counteracted the enhancement of release caused by the phorbol esters. Since the relative inhibitory effect of polymyxin B remained unchanged a functional antagonism and not a competitive one was assumed (Feuerstein *et al.*, 1987; Versteeg & Ulenkate, 1987). The same conclusion can be drawn from the present data in regard to acetylcholine release. However, the mechanism by which polymyxin B antagonizes the phorbol ester-induced facilitation of transmitter release requires further investigation.

In contrast to the action of polymyxin B the net effect of inhibition, i.e. the absolute reduction of tritium overflow caused by the muscarine receptor agonists, oxotremorine and carbachol, was not changed in the presence of 4β-PDB (Table 5). In addition, transmitter release was increased by 4β-PDB and atropine in an additive manner, both under conditions of weak or strong (when acetylcholine esterase was inhibited) autoinhibition (Figure 2). Each drug was used at or near to a concentration that caused maximum facilitation of release. Both the unchanged inhibitory net effects of the muscarine receptor agonists as well as the additive nature of the effects of 4β-PDB and atropine exclude the possibility that the enhancement of release was due to an inactivation of the autoreceptor-coupled inhibitory feedback system by the phorbol ester. In addition, a direct participation of protein kinase C in the post muscarine receptor mechanism seems to be very unlikely. Similar interpretation of experimental data concerning autoreceptor-mediated modulation of acetylcholine release in rat hippocampus was given by Versteeg & Florijn (1987), while this paper was in preparation.

Similar results have been obtained from experiments where phorbol ester effects on autoreceptor-mediated modulation of stimulated [^{3}H]-noradrenaline (Allgaier *et al.*, 1986; 1987; Versteeg & Florijn 1987) and [^{3}H]-5-hydroxytryptamine (Feuerstein *et al.*, 1987) release from hippocampal slices were studied.

Inhibition of acetylcholine release by the heteroreceptor agonists (−)-PIA (Table 7) and EKC (Table 8) also appears not to be directly affected by protein kinase C activation, since similar inhibitory effects of both agonists were seen, irrespective of whether the phorbol ester was present or absent. From these and former results (Allgaier *et al.*, 1986; 1987; Feuerstein *et al.*, 1987; Versteeg & Florijn, 1987) it can be assumed that, while protein kinase C generally participates in the modulation of the evoked transmitter release, this enzyme is not directly linked to mechanisms mediating inhibition of release via presynaptic receptors.

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