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Basal and electrically stimulated release of [^{3}H]noradrenaline and [^{3}H]dopamine from rat amygdala slices in vitro: effects of 4β-phorbol 12,13-dibutyrate, 4α-phorbol 12,13-didecanoate and polymyxin B

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The protein kinase C activator 4β-phorbol 12,13-dibutyrate (PDB) enhanced in a concentration-dependent manner the electrically stimulated release of [^{3}H]noradrenaline ([^{3}H]NA) and [^{3}H]dopamine ([^{3}H]DA) from rat amygdala slices in vitro. PDB enhanced the basal release of [^{3}H]NA and [^{3}H]DA as well. 4α-Phorbol 12,13-didecanoate, which lacks the capacity to activate protein kinase C, was without effect on either basal or electrically stimulated release of [^{3}H]NA and [^{3}H]DA. Polymyxin B, which is a relatively selective protein kinase C inhibitor, decreased in a concentration-dependent manner the electrically stimulated release of both [^{3}H]NA and [^{3}H]DA from amygdala slices, whereas it enhanced the basal release of both neuromessengers. In the presence of 1.5×10^{-7} M PDB, a concentration which when added to the superfusion medium alone doubled the electrically stimulated release of both [^{3}H]NA and [^{3}H]DA, polymyxin B again decreased in a concentration-dependent manner the release of both neuromessengers. At all polymyxin B concentrations used, the effect of the PKC inhibitor, expressed as percent inhibition, in the presence of PDB was approximately the same as that observed in the absence of PDB. This suggests that the antagonism between PDB and polymyxin B at the level of protein kinase C is not a competitive one. The effects of PDB and polymyxin B on basal release were additive. Taken together, these data suggest that in the amygdala presynaptically localized protein kinase C plays a role in signal transduction processes related to the exocytotic secretion of NA and DA from their nerve terminals.

Protein kinase C is acting as mediator between the activation of membrane receptors and intracellular responses[30] (for recent reviews see refs. 1, 18, 26). Tumor promoting phorbol esters, by activating protein kinase C, mimick the effects of the endogenous protein kinase C activator diacylglycerol[9]. Based on the effects of phorbol esters, it has been concluded that the diacylglycerol/protein kinase C pathway is involved in the stimulus/secretion process of various hormones and neuromessengers from endocrine and neuronal tissues (for references see ref. 26). Phorbol esters have been shown to enhance the release of somatostatin[25] and dopamine[37] from fetal brain neurons in culture. Recently, it was reported that phorbol esters stimulate the depolarization-induced release of acetylcholine from caudate nucleus slices[31] and of noradrenaline[2,3,34] and acetylcholine and serotonine[34] from hippocampal slices, which suggests a role of presynaptically localized protein kinase C in the stimulus/secretion process of these neuromessengers from their terminals in these brain regions.

We report here that the electrically evoked release of both [^{3}H]noradrenaline ([^{3}H]NA) and [^{3}H]dopamine ([^{3}H]DA) from rat amygdala slices in vitro was enhanced by the protein kinase C activator 4β-phorbol 12,13-dibutyrate (PDB) and reduced by the protein kinase C inhibitor polymyxin B, whereas the 4α-phorbol ester 4α-phorbol 12,13-didecanoate (PDD) was without effects. This indicates that also in the amygdala presynaptically localized protein kinase C plays a role in cell surface transduction related to the exocytotic secretion of NA and DA from their nerve terminals.

To study the effects of PDB, PDD and polymyxin

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B on basal and electrically stimulated release of [^{3}H]NA and [^{3}H]DA from amygdala tissue, an in vitro method was used as described by Stoof et al.[29] and Schoffelmeer et al.[27] with slight modifications[34].

Male Wistar rats, weighing 150–180 g, were killed by decapitation. The brains were excised rapidly. Subsequently, the amygdala was dissected bilaterally from the surrounding tissue from a 2 mm thick coronal section extending from 1.5 to 3.5 mm caudal of the commissura anterior. The tissue pieces, weighing about 15 mg per rat, were chopped into slices of 0.3 × 0.3 × 2.0 mm, using a McIlwain tissue chopper. The chopped tissue was then transferred to a beaker containing carbogenated Krebs–Ringer bicarbonate medium, pH 7.4. Typical time between decapitation of the rat and transfer of the chopped tissue to the medium was 100 s. The composition of the medium was (mM): NaCl 121, KCl 1.87, K_2HPO_4 1.17, $MgSO_4$ 1.17, $CaCl_2$ 1.22, $NaHCO_3$ 20 and glucose 11.1. The slices were preincubated for 10 min at 37 °C in a metabolic shaker under a 95% O_2–5% CO_2 atmosphere. To the incubation medium was then added either 5 μCi [7,8-^{3}H]noradrenaline (spec. act. 32 Ci·mmol^{-1}) or 5 μCi [1,2-^{3}H]dopamine (spec. act. 40 Ci·mmol^{-1}). The incubation was then continued for another 15 min to enable labelling of the neuromessenger pools in the tissue. In order to inhibit the uptake of [^{3}H]NA in dopaminergic terminals and of [^{3}H]DA in noradrenergic terminals, GBR 12909 (1-[2-[bis(4-fluorphenyl)methoxy]ethyl]-4-(3-phenylpropyl)-piperazine dihydrochloride; 5×10^{-7} M) or desipramine (3×10^{-6} M), respectively, were added to the incubation media. These compounds were also present in the superfusion media during the subsequent superfusion procedures.

After washing the slices gently with fresh medium two times, approximately 5 mg of the labelled tissue were transferred to each of 24 superfusion chambers (volume 0.25 ml) maintained at 37 °C and superfused at a rate of 0.25·ml^{-1} with medium. After 40 min of superfusion (t = 40 min), three 15-min fractions were collected: a prestimulus fraction from t = 40–55 min; a fraction during which the slices were subjected to electrical stimulation from t = 55–70 min; and, a post-stimulus fraction from t = 70–85 min. The electrical stimulation (biphasic block-pulses, 24 mA, 2 ms, 2 Hz) lasted from t = 55–65 min. These stimulation conditions were chosen based on the results of frequency–response experiments, which showed a maximal stimulation of the release of both [^{3}H]NA and [^{3}H]DA at a frequency of 10 Hz and an approximately half-maximal stimulation at 2 Hz (data not shown). Phorbol esters were present in the superfusion medium from t = 35 min. In experiments in which polymyxin B and PDB were present in the superfusion medium together, polymyxin B was added at t = 20 min and PDB at t = 35 min. At the end of the superfusion (t = 85 min), i.e. after the collection of the poststimulus fraction, the radioactivity remaining in the tissue was extracted in 0.1 N HCl. The radioactivity in the superfusion fractions and in the tissue was quantified by liquid scintillation spectrometry.

The release of ^{3}H radioactivity in each fraction was calculated as the fractional rate of the total radioactivity in the tissue at the beginning of the collection of the fraction. The fractional rate of the release of radioactivity in the prestimulation fraction (t = 40–55 min) was taken as a measure of basal release. The electrically stimulated release was calculated as release in excess of basal release by subtracting the means of the fractional rates of the pre- and poststimulation fractions from the fractional rate of the stimulation fraction. The results are expressed as percentage of controls (no phorbol esters or polymyxin B added to the superfusion medium). Statistical analysis of the data was performed by one-way ANOVA, followed by Student–Newman–Keuls tests. A P-value of less than 0.05 was taken as indicating a significant difference.

The phorbol esters, polymyxin B sulfate and desipramine were purchased from Sigma; [^{3}H]NA and [^{3}H]DA from Amersham. GBR 12909 was kindly donated by Dr. W. Hespe, Gist-Brocades NV, Haarlem, The Netherlands.

In preliminary experiments it was ascertained that dimethyl sulfoxide, used as solvent for PDB and PDD, did not cause a significant alteration of either the basal or the electrically evoked release of [^{3}H]NA and [^{3}H]DA in the concentrations used (up to 0.05% in the superfusion medium at the highest concentrations of the phorbol esters; data not shown).

Table I shows that the 4β-phorbol ester PDB enhanced the basal efflux of both [^{3}H]NA and [^{3}H]DA from amygdala slices in vitro in a concentration-dependent manner. A significant effect on basal efflux

TABLE I

Effects of the 4β-phorbol ester PDB and of the 4α-phorbol ester PDD on basal release of [^{3}H]NA and [^{3}H]DA from rat amygdala slices in vitro

For details concerning the method and calculations: see text. Each value is the mean ± S.E.M. of 8–20 observations obtained in 2–5 separate experiments. Absolute control values for the fractional rates of basal release (means ± S.E.M.) were: 2.57 ± 0.13 ([^{3}H]NA, $n = 20$) and 3.84 ± 0.06 ([^{3}H]DA, $n = 20$).

Concentration phorbol ester (M)	*Basal release of [^{3}H]NA (% of controls)*		*Basal release [^{3}H]DA (% of controls)*	
	PDB	*PDD*	*PDB*	*PDD*
0	99.8 ± 1.3	99.7 ± 1.7	99.8 ± 1.3	100.0 ± 1.4
3×10^{-9}	98.5 ± 1.4	–	101.9 ± 1.2	–
10^{-8}	99.9 ± 0.9	98.5 ± 1.6	112.6 ± 2.8	99.6 ± 2.4
3×10^{-8}	115.6 ± 2.9*	–	132.2 ± 4.4*	–
10^{-7}	122.9 ± 2.7*	99.0 ± 3.6	128.2 ± 5.0*	100.3 ± 2.2
3×10^{-7}	130.6 ± 2.7*	–	143.8 ± 8.9*	–
10^{-6}	130.3 ± 4.8*	107.1 ± 13.8	163.1 ± 7.6*	107.3 ± 4.9

* $P < 0.05$ for difference with controls (no phorbol ester present in the superfusion medium) (ANOVA, followed by Student–Newman–Keuls tests).

of ^{3}H radioactivity was found at a PDB concentration of 3×10^{-8} M. At the highest concentration (10^{-6} M) the increase in [^{3}H]NA release was 30% over control values; that of [^{3}H]DA release amounted to 63%. In contrast, the 4α-phorbol ester PDD did not affect the basal efflux of either [^{3}H]NA or [^{3}H]DA at any of the concentrations used (Table I). The electrically stimulated release of [^{3}H]NA as well as that of [^{3}H]DA was significantly stimulated by PDB in concentrations of 10^{-8} M and higher (Fig. 1). When PDB was present in the superfusion medium in a concentration of 3×10^{-7} and 10^{-6} M, a maximal increase of 100–120% in electrically stimulated release was observed (Fig. 1). At none of the concentrations used PDD had any effect on the electrically stimulated release of either [^{3}H]NA or [^{3}H]DA (Fig. 1).

Polymyxin B enhanced in a concentration-dependent manner the basal efflux of both [^{3}H]NA and [^{3}H]DA (Fig. 2A); at the same time it caused a concentration-dependent decrease in the electrically stimulated release of both neuromessengers (Fig. 2B). The effects were significant ($P < 0.05$, ANOVA followed by Student–Newman–Keuls tests) at polymyxin B concentrations of 240 units·ml^{-1} and higher, except for that on basal [^{3}H]NA release, which reached significance at a polymyxin B concentration of 720 units·ml^{-1} (Fig. 2).

Fig. 2 also shows the effects of increasing concentrations of polymyxin B on basal and electrically stimulated [^{3}H]NA and [^{3}H]DA release in the presence of 1.5×10^{-7} M PDB. As can be seen from the curves in Fig. 2B, PDB in this concentration approximately doubled the electrically stimulated release of both neuromessengers, both when added to the superfusion medium alone and when polymyxin was

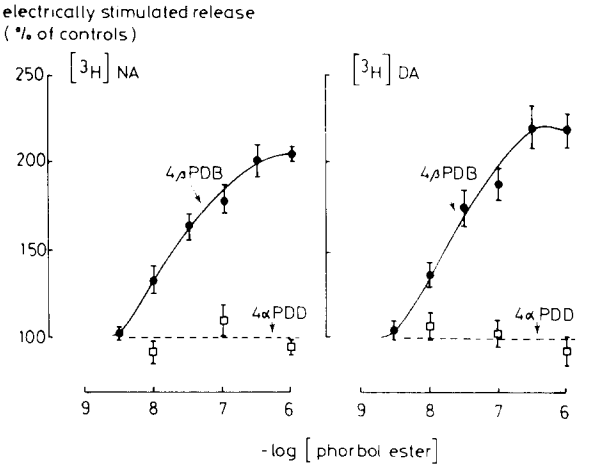

Fig. 1. Effects of increasing concentrations of the 4β-phorbol ester PDB (●) and the 4α-phorbol ester PDD (□) on the electrically stimulated release of [^{3}H]NA and [^{3}H]DA from rat amygdala slices in vitro. For details concerning the method and calculations: see text. Each value is the mean ± S.E.M. of 8–20 observations obtained in 2–5 separate experiments. Absolute control values for the fractional rates of the electrically stimulated release in excess to basal release (means ± S.E.M.) were: 3.93 ± 0.28 ([^{3}H]NA, $n = 20$) and 4.27 ± 0.30 ([^{3}H]DA, $n = 20$).

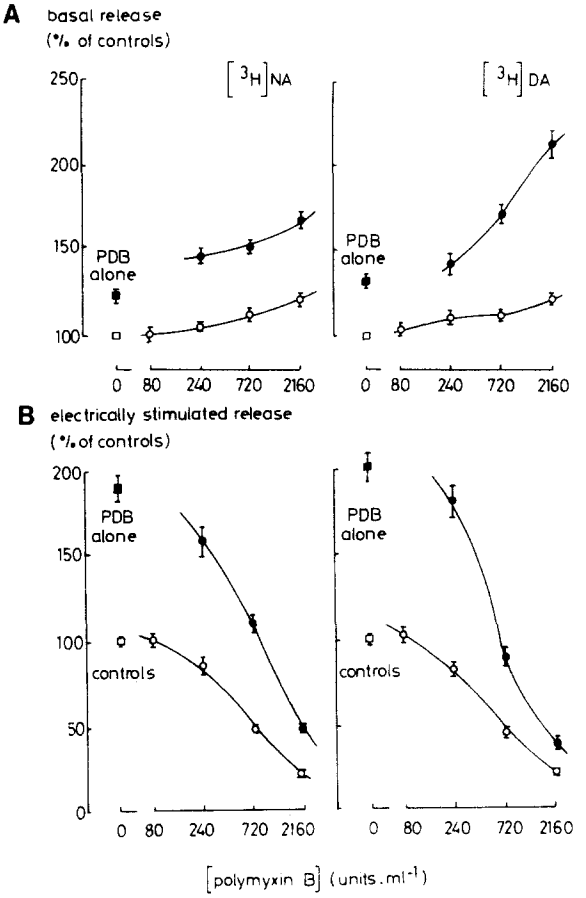

Fig. 2. Effects of increasing concentrations of polymyxin B alone (○) and in the presence of the 4β-phorbol ester PDB (1.5×10^{-7} M, ●) on basal release (A) and on electrically stimulated release (B) of [^{3}H]NA (left panels) and [^{3}H]DA (right panels) from amygdala slices in vitro. Controls (□, no drugs in superfusion medium) and the effects of PDB, 1.5×10^{-7} M, alone (■) are also shown. For details concerning the method and calculations: see text. Each value is the mean ± S.E.M. of 12–24 observations obtained in 3–6 separate experiments (open symbols) or 8–12 observations obtained in 3 separate experiments (closed symbols).

also present. In other words, at any of the polymyxin B concentrations used the effect of this compound, expressed as percent inhibition, in the presence of PDB was the same as that observed in the absence of PDB. The enhancing effects of PDB and polymyxin B on basal release were additive (Fig. 2A).

The amygdala is a limbic brain structure which participates in the modulation and regulation of autonomic functions and behavior (for a review see ref. 19). NA and DA have an important role in amygdaloid function. This follows from the results of experiments in which the effects were studied of microinjections of adrenoceptor agonists and antagonists into the amygdala on behavior[12–14,20,28], as well as from experiments correlating catecholamine metabolism in the amygdala and the performance of rats in operant or passive avoidance behavior[4,16,17,21,24]. Results of lesion studies and of microinjection experiments support the notion that the amygdala is the anatomical substrate for the antiamnestic effects of vasopressin and related neuropeptides[7] and of ACTH-like peptides on avoidance behavior[33]. Both cell bodies and projections of the vasopressin system[8,22] as well as projections of the pro-opiomelanocortin system[10] have been observed in the amygdala, while a high density of vasopressin binding sites has been found in this brain structure[6,11]. An interaction of vasopressin and DA at the level of the amygdala has been reported[32].

The present experiments are the first of a series in which we intend to investigate whether vasopressin, which is most probably acting via vasopressin receptors, and peptides of the ACTH/MSH-family, which have been shown to have protein kinase C-inhibiting effects (for a review see ref. 15), interact with catecholaminergic mechanisms in the amygdala at the level of the release process. In the present study we used PDB, which has been described to directly activate protein kinase C, thereby mimicking the effects of the second messenger diacylglycerol[9] (for reviews see refs. 1, 18, 26), and polymyxin B, which is a relatively selective inhibitor of protein kinase C[23,36], to investigate whether or not the diacylglycerol/protein kinase C pathway is involved in the stimulus/secretion process of NA and DA from their terminals in the amygdala. It appeared that the 4β-phorbol ester PDB in a concentration-dependent manner enhanced the electrically stimulated release of both [^{3}H]NA and [^{3}H]DA from preloaded amygdala slices in vitro, whereas the 4α-phorbol ester PDD, which lacks protein kinase C-activating properties[5], did not. The finding that at concentrations of PDB higher than 10^{-7} M there is no further increase in release, is probably due to the enhanced activation of autoreceptors as a consequence of the approximately doubled release of the neuromessengers at these concentrations. At present, experiments are being carried out to establish the presence and type of these autoreceptors in the amygdala.

Further evidence for a role of protein kinase C in

the stimulus/secretion process of NA and DA from their terminals in the amygdala was obtained in experiments in which polymyxin B was added to the superfusion medium alone and in combination with PDB. Polymyxin B reduced the electrically evoked release of radiolabelled NA and DA from amygdala slices in a concentration-dependent manner. A slight, but significant increasing effect on basal efflux of radioactivity was also evident in the presence of polymyxin B. The concentrations of polymyxin B needed to significantly affect NA and DA release, though rather high, were of the same order of magnitude as those found to be effective by Allgaier and Hertting[2] and Wakade et al.[35] on the release of NA by rabbit hippocampal slices and catecholamines by rat adrenal medulla respectively.

The results of the experiments in which polymyxin B was added to the superfusion medium together with PDB show that there is antagonism between the effects on the electrically stimulated release of [^{3}H]NA and [^{3}H]DA of PDB and polymyxin B. This antagonism, which was evident from a concentration-dependent reduction of the effects of PDB on the release of both neuromessengers in the presence of polymyxin B, is most likely at the level of protein kinase C[23,36]. Interestingly, it was found that, at any of the polymyxin B concentrations used, the effect of this protein kinase C inhibitor, expressed as percent inhibition, in the presence of PDB was the same as that observed in the absence of PDB. This suggests that the antagonisms between polymyxin B and PDB at the level of protein kinase C is not a competitive one.

Previously, it has been reported that protein kinase C is involved in the stimulus/secretion process of somatostatin[25] and dopamine[37] from brain neurons in culture, of acetylcholine from caudate nucleus slices[31] and of noradrenaline[2,3,34] and serotonin and acetylcholine[34] from hippocampal slices in vitro. The present data add NA and DA in the amygdala to this list. Future research should provide evidence for the nature of the presynaptic receptors which via diacylglycerol/protein kinase C pathways are linked to these stimulus/secretion processes.

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