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Participation of Protein Kinase C and Regulatory G Proteins in Modulation of the Evoked Noradrenaline Release in Brain

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SUMMARY

1. In the present paper two questions are discussed: (A) Does protein kinase C (PKC) participate in the modulation of evoked noradrenaline release in brain tissue? and (B) Is there any link between presynaptic α_2-adrenoceptors and regulatory G proteins?

2. Slices of the middle part of the rabbit hippocampus, labeled with ^{3}H-noradrenaline, were superfused with medium containing the reuptake inhibitor cocaine. During superfusion the tissue was stimulated twice electrically for 2 min each.

3. The PKC activators 4β-phorbol 12,13-dibutyrate (4β-PDB) and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) increased the stimulation-evoked transmitter release in a concentration-dependent manner. 4α-PDB and 4-*O*-methyl-TPA, which do not activate PKC, were without effect on transmitter release. Polymyxin B, an inhibitor of PKC, diminished the stimulus-evoked overflow and counteracted the effects of the phorbol esters. The increases in release caused by phorbol esters and the α_2-adrenoceptor antagonist yohimbine were additive.

4. Treatment of hippocampal tissue with islet-activating protein (IAP) or *N*-ethylmaleimide (NEM), both known to inactivate the regulatory G proteins Gi and Go by chemical modification, led to a marked increase in evoked

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noradrenaline release. In addition, the effects of both the α_2-adrenoceptor agonist clonidine and the α_2-adrenoceptor antagonist yohimbine were inhibited.

5. The facilitatory effects of IAP and NEM on transmitter release were not additive. In synaptosomes prepared from rabbit hippocampus two polypeptides with molecular weights corresponding to those of α_i and α_o were ^{32}P-ADP-ribosylated with IAP. Pretreatment of synaptosomes with NEM reduced the subsequent ADP ribosylation by IAP concentration dependently.

6. The above results suggest that PKC is involved in the modulation of noradrenaline release in the rabbit hippocampus. The presynaptic α_2-autoreceptors modulate transmitter release by a mechanism which is not directly affected by PKC. The α_2-autoreceptor-mediated signals seem to be transduced across the plasma membrane via regulatory G proteins.

INTRODUCTION

In various cell types Ca^{2+}-dependent exocytosis was augmented by activation of protein kinase C (PKC) (Nishizuka, 1984). The physiological activator of PKC is diacylglycerol, which is transiently produced by enzymatic cleavage of inositol-phospholipids (Nishizuka, 1984; Berridge, 1984). Activation of PKC can be mimicked by tumor-promoting phorbol esters such as 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and 4β-phorbol 12,13-dibutyrate (4β-PDB). Since TPA enhanced the evoked ^{3}H-dopamine release from neuronal cells (Zurgil and Zisapel, 1985), it was assumed that PKC is also involved in depolarization-induced exocytotic release of neurotransmitters.

Evoked transmitter release is often modified by receptors localized on or near to the nerve terminals of a neuron, so-called presynaptic receptors (Starke, 1981). Nerve endings of noradrenergic neurons are equipped with α_2-adrenoceptors. By activation of these receptors the action potential-induced release of noradrenaline is reduced. As these receptors become operative by concentrations of the endogenous transmitter occurring under physiological stimulation frequencies, this autoinhibitory feedback mechanism is of physiological relevance. The extent of autoinhibition depends on the biophase concentration of the endogenous neurotransmitter: at a lower firing rate of the neuron, autoinhibition is less pronounced than at higher stimulation frequencies.

Little is known about the biochemical mechanisms following activation of presynaptic α_2-adrenoceptors. Since depolarization-induced noradrenaline release is Ca^{2+} dependent it was tempting to assume that the receptor-mediated inhibition of transmitter release limits the access of Ca^{2+} to the mechanism of stimulus–secretion coupling. Adenylate cyclase appears to facilitate noradrenaline release both in the periphery and in the central nervous system (Mulder *et al.*, 1984; Starke, 1987). In many cells α_2-adrenoceptors have been shown to inhibit adenylate cyclase via the regulatory guanine nucleotide-binding protein Gi (Jakobs *et al.*, 1984). In brain the existence of two G proteins, Gi and Go, has been demonstrated (Sternweis and Robishaw, 1984). Both proteins can be

biologically inactivated by chemical modification of their α-subunits by islet-activating protein (IAP) or *N*-ethylmaleimide (NEM) (Katada and Ui, 1982; Asano and Ogasawara, 1985).

Therefore, it seemed to be of interest to study if PKC is involved in the modulation of noradrenaline release and if regulatory nucleotide-binding proteins participate in the autoinhibitory feedback mechanism. Studies were performed on slices of the rabbit hippocampus. As during preparation of the tissue slices noradrenergic nerve terminals were cut off from their cell bodies located in the locus coeruleus, presynaptic mechanisms underlying modulation of transmitter release could be directly investigated.

METHODS

Slices of the middle part of the rabbit hippocampus (0.35 mm) were prelabeled with ^{3}H-noradrenaline, superfused (1 ml/min) with a modified Krebs–Henseleit medium containing the reuptake inhibitor cocaine (30 μM). During superfusion the slices were stimulated electrically twice for 2 min each. The radioactivity of 5-min collection periods and the remaining radioactivity of the tissue slices at the end of the experiment were determined (Allgaier *et al.*, 1985). The fractional rate of tritium outflow/5 min was calculated as (tritium outflow/5 min)/(tritium content in the slice at the start of the respective 5-min collection period). The electrically evoked overflow of tritium (S_1 or S_2, expressed as a percentage of tissue tritium at the beginning of the respective stimulation period) was calculated by subtraction of the basal outflow from the total overflow of radioactivity.

All results are shown as arithmetic means ±SE. The significance of differences 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.

RESULTS AND DISCUSSION

Participation of Protein Kinase C in Modulation of Noradrenaline Release

The stimulation-evoked tritium overflow from rabbit hippocampal slices, prelabeled with ^{3}H-noradrenaline, ressembles physiological action potential-induced noradrenaline release (Jackisch *et al.*, 1984). Both 4β-PDB (0.01–10 μM) and TPA (0.3–30 μM) increased the electrically evoked noradrenaline release in a concentration-dependent manner without affecting basal outflow. Phorbol esters which do not activate PKC, such as 4α-PDB and 4-*O*-methyl-TPA, did not influence transmitter release (Allgaier *et al.*, 1986a, 1987b). Polymyxin B, an inhibitor of protein kinase C (Kuo *et al.*, 1983), diminished the evoked noradrenaline release in a time- and concentration-dependent manner. In addition, polymyxin B counteracted the facilitatory effects of 4β-PDB (Allgaier and Hertting, 1986).

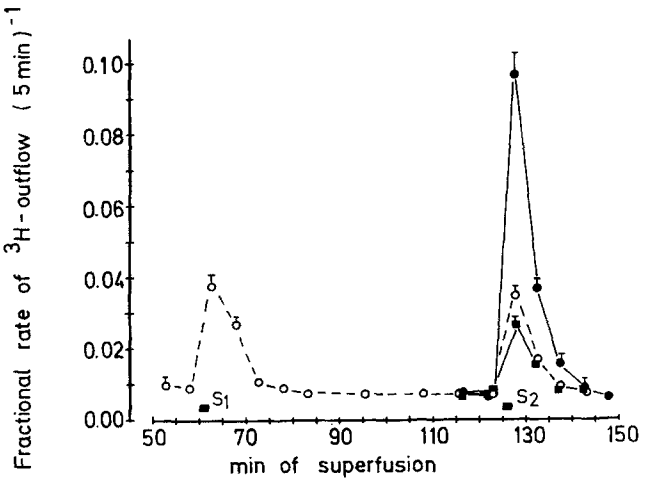

Fig. 1. Effects of 4β-phorbol 12,13-dibutyrate (4β-PDB) and polymyxin B on basal and on electrically evoked outflow of tritium from slices of the rabbit hippocampus, prelabeled with ^{3}H-noradrenaline. During superfusion with medium containing cocaine (30 μM), slices were stimulated twice electrically (S_1, S_2; 3 Hz, 24 mA, 2 msec, 5 V/cm) for 2 min each. Drugs were given from 15 min before S_2 onward. Fractional rates of tritium outflow are shown as means ±SE. S_2/S_1 ratios of the overflow were as follows: 1.00 ± 0.04, controls (○), $N = 6$; 3.05 ± 0.04, 4β-PDB (1 μM ●), $N = 6$; 0.74 ± 0.06, polymyxin B (100 μM; ■), $N = 4$. N = number of experiments.

A typical superfusion experiment is depicted in Fig. 1. Both drugs, 4β-PDB and polymyxin B, were added 15 min before the second stimulation period to the superfusion medium. 4β-PDB (1 μM) increased the evoked overflow by about 200%, whereas polymyxin B (100 μM) inhibited it by about 25% (S_2/S_1 expressed as a percentage of control; see legend to Fig. 1). Prolongation of the exposure to polymyxin B up to 45 min led to an inhibition of about 50% (Allgaier and Hertting, 1986). These observations suggest a participation of PKC in the chain of events of stimulus–secretion coupling.

What mechanism of action can serve as an explanation of the increase in noradrenaline release following PKC activation? The increase in the evoked tritium overflow induced by activation of PKC cannot be explained by an inhibition of the reuptake of ^{3}H-noradrenaline, since all experiments were performed in the presence of the uptake inhibitor cocaine. Moreover, under our experimental conditions complete blockade of the uptake increased the evoked tritium overflow only from about 2 to 4% of the tissue tritium content at the onset of stimulation, whereas 4β-PDB (1 μM) raised the overflow up to 12%.

At the stimulation frequency used in our experiments a strong autoinhibition was present, as demonstrated by the fact that blockade of the α_2-autoreceptors by the specific α_2-adrenoceptor antagonist yohimbine (1 μM) increased transmitter release to about 22% of tissue tritium. The combined administration of 4β-PDB and yohimbine increased the overflow up to 36%. As the effects of the phorbol

ester and yohimbine were additive or even overadditive (Allgaier *et al.*, 1987b), it can be deduced that PKC activation did not directly interfere with the autoinhibitory mechanism. However, phorbol esters influence autoreceptor-mediated effects indirectly by changes in the biophase concentration of the endogenous transmitter (Allgaier *et al.*, 1987b).

The enhancement of release could be explained by an action of PKC on Ca^{2+} or K^+ channels. It has been shown that K^+ channels were blocked by phorbolesters in hippocampal pyramidal cells, thereby slowing depolarization (Baraban *et al.*, 1985; Storm, 1987). A broadening of the repolarization period in noradrenergic nerve terminals would enhance noradenaline release.

The effects of phorbol esters on Ca^{2+} channels are contradictory; both activation of Ca^{2+} currents, which would increase transmitter release, and inhibition of Ca^{2+} fluxes have been reported (DeRiemer *et al.*, 1985; DiVirgilio *et al.*, 1986; Wakade *et al.*, 1986).

The available data point to a participation of PKC in the modulation of noradrenaline release, while this enzyme is not directly involved in the α_2-autoinhibitory mechanism. Similar results have been reported recently by Veersteg and Florijn (1987). A final decision concerning the mechanism underlying the modulatory role of PKC can not be reached as yet.

Linkage of Presynaptic α_2-Autorecetors to a Regulatory G Protein

Activation of postsynaptically located α_2-adrenoceptors inhibits the catalytic activity of adenylate cyclase. As the receptor-mediated inhibition of adenylate cyclase involves Gi, the inhibitory action of α_2-adrenoceptor agonists on adenylate cyclase activity was diminished by IAP or by NEM, e.g., in adipocytes and platelets (Jakobs *et al.*, 1984). Indirect evidence for the involvement of a regulatory G protein in modulation of noradrenaline release by presynaptic α_2-adrenoceptors was provided by Lai *et al.* (1983) from experiments with IAP performed on guinea pig was deferens.

Preincubation of hippocampal slices with IAP increased the electrically evoked noradrenaline release in a time (Fig. 2) and concentration-dependent manner. Basal outflow was not affected. Effects of IAP on noradrenaline release were seen only after an incubation period of at least 6 hr (Allgaier *et al.*, 1985). Such a long lag phase is explainable by the fact that IAP has to penetrate the membrane before a chemical modification of G protein can take place. Rather high concentrations of IAP were used (up to 8 μg/ml), compared to those in experiments performed on cells. Diffusion of IAP to the presynaptic site of action seems to be critical in tissue slices. However, comparable (Lai *et al.*, 1983) or even higher (Martinez-Olmedo and Garcia-Sáinz, 1984) concentrations of IAP were used from other groups working on tissue preparations.

NEM influenced the evoked noradrenaline release in a manner similar to that of IAP. However, incubation periods with NEM of 30 min were sufficient to get an increase in release by about 100%. In contrast to IAP basal outflow was also increased by NEM. This may be due to the more unspecific action of NEM compared to that of IAP (Allgaier *et al.*, 1986*b*).

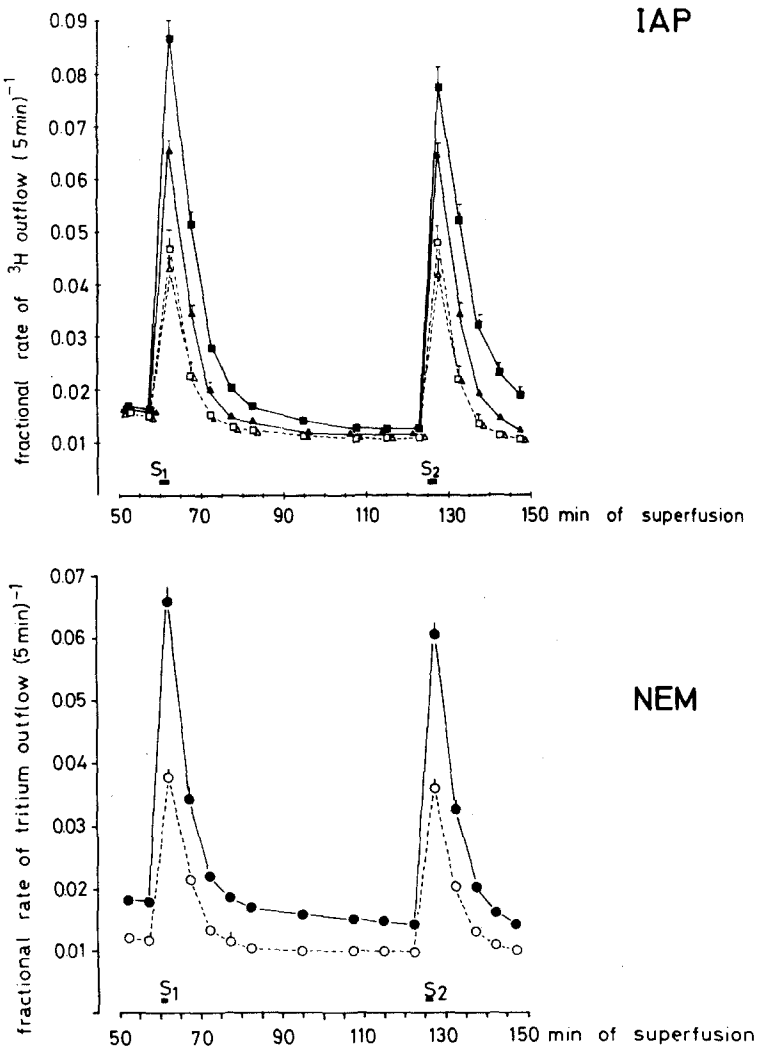

Fig. 2. Effects of IAP and NEM on basal and on stimulation-evoked outflow of tritium from hippocampal slices, labeled with ^{3}H-noradrenaline. Slices were pretreated either with IAP (8 μg/l) for 12 hr (▲) or 18 hr (■) or with NEM (30 μM; ●) for 30 min before labeling with ^{3}H-noradrenaline. Control slices (open symbols) were incubated under the same conditions but in the absence of IAP or NEM (Allgaier *et al.*, 1985, 1986b). After ^{3}H-labeling slices were superfused with medium containing cocaine (30 μM) and stimulated twice electrically for 2 min each (3 Hz, 2 msec, 24 mA, 5 V/cm). Means ± SE of the fractional rates of tritium outflow are given. Number of experiments per group, 6–11.

Table I. Effects of IAP and NEM on the Actions of Clonidine and Yohimbine on Stimulation-Evoked Noradrenaline Release[a]

Drug before S_2 (μM)	IAP or NEM	$S_2/S_1 \pm SE$	% of control	N
—	—	1.17 ± 0.06	—	8
Clonidine (0.1)	—	0.43 ± 0.02	37	6
Yohimbine (1.0)	—	3.95 ± 0.08	338	4
—	IAP	1.02 ± 0.06	—	8
Clonidine (0.1)	IAP	0.93 ± 0.03	91	4
Yohimbine (1.0)	IAP	1.73 ± 0.08	170	4
—	—	1.02 ± 0.02	—	8
Clonidine (0.1)	—	0.32 ± 0.02	31	4
Yohimbine (1.0)	—	4.19 ± 0.10	410	4
—	NEM	1.03 ± 0.02	—	6
Clonidine (0.1)	NEM	0.70 ± 0.02	68	4
Yohimbine (1.0)	NEM	2.67 ± 0.11	259	4

[a] Slices were pretreated either with IAP (8 μg/ml) for 18 hr or with NEM (30 μM) for 30 min, then labeled with ^{3}H-noradrenaline. Control slices were incubated under the same conditions but in the absence of IAP or NEM (Allgaier *et al.*, 1985, 1986b). After ^{3}H-labeling slices were superfused with medium containing cocaine (30 μM) and stimulated twice electrically for 2 min each (S_1, S_2; 3 Hz, 2 msec, 24 mA, 5 V/cm). Clonidine or yohimbine was added to the superfusion medium from 15 min before S_2 onward. S_2/S_1 represents the ratio of the overflow evoked by the two stimulation periods. N = number of experiments.

The increase in the evoked noradrenaline release by both IAP and NEM is compatible with the explanation of an impairment of the autoinhibitory feedback mechanism at the level of a G protein. In this case the effects of α_2-adrenoceptor agonists and antagonists should be also reduced by IAP and NEM.

It can be seen from Table I that indeed both the facilitatory effects of yohimbine and the inhibitory effects of clonidine were strongly reduced by either IAP or NEM.

It can be argued that the SH-reagent NEM influences noradrenaline release by additional unspecific actions. However, some experimental data prove NEM as a valuable tool for exploring the functional role of G proteins in neurotransmitter release: (1) receptor protection experiments showed that NEM did not alkylate the active site of the receptor (Allgaier *et al.*, 1986b); (2) the effects of IAP and NEM were not additive (Hertting *et al.*, 1986); and (3) in synaptosomes prepared from rabbit hippocampus, NEM diminished the IAP-induced ^{32}P-ADP ribosylation of the α-subunits of Gi and Go in a concentration-dependent manner (Fig. 3).

Taken together the results obtained with IAP and NEM indicate the participation of a regulatory G protein in the modulation of noradrenaline release by α_2-autoreceptors. Moreover, G proteins seem to participate similarly in the inhibition of noradrenaline release mediated by presynaptic heteroreceptors, such as A_1-adenosine (Fredholm and Lindgren, 1986, 1987; Allgaier *et al.*, 1987a) and κ-opioid receptors (Allgaier *et al.*, 1987c). In addition, G proteins are involved in receptor-mediated modulation of release of various transmitters, such as glutamate (Dolphin and Prestwich, 1985) and acetylcholine (Hertting *et al.*, 1987). From the present data it is not possible to decide which G protein, Gi or Go or both, is

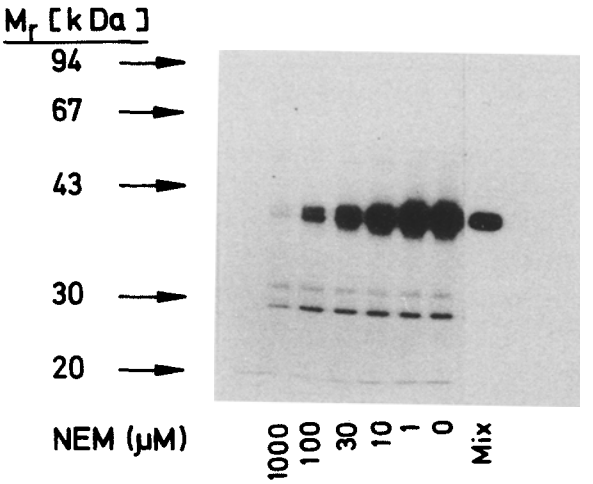

Fig. 3. ^{32}P-ADP ribosylation of hippocampal synaptosomes pretreated with NEM. Synaptosome fractions 3 and 4, isolated by Percoll gradient centrifugation (Robinson and Lovenberg, 1986), were resuspended in a Krebs–Ringer buffer (KRB) and adjusted to a protein concentration of 2250 μg/ml. One hundred microliters of this suspension was added to 100 μl of KRB containing NEM (final concentration of NEM as indicated). NEM treatment, carried out at 37°C, was stopped after 30 min by the addition of dithiothreitol. Synaptosomes were recovered by centrifugation. The pellet was resuspended in 50 m*M* Tris/HCL, 1 m*M* EDTA, pH 8.0. IAP-induced ^{32}P-ADP ribosylation of synaptosomal proteins was performed using a method based on that of Kawai *et al.* (1986). Samples containing 150 μg of protein were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis on a 10% gel as described by Laemmli (1970). Subsequently, autoradiography was performed. The right lane represents the autoradiogram of a mix of purified Gi and Go (5 μg).

involved in the modulation of transmitter release. However, there seems to be an interaction of the auto- and heteroreceptor-coupled signal transduction mechanisms of a nerve terminal, since inhibition of release by heteroreceptor agonists depended on the extent of autoreceptor occupation (Allgaier *et al.*, 1987a, c).

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