Evidence for a Presynaptic Adenylate Cyclase System Facilitating [³H]Norepinephrine Release from Rat Brain Neocortex Slices and Synaptosomes

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Abstract

The effects of drugs known to enhance intracellular cyclic AMP levels on depolarization-induced [³H]norepinephrine release from superfused rat neocortical slices and synaptosomes were investigated. The adenylate cyclase activator forskolin, the membrane-permeating cyclic AMP analogues 8-bromo-cyclic AMP and dibutyryl cyclic AMP, as well as the phosphodiesterase inhibitors isobutylmethylxanthine and 4-(3-cyclopentylloxy-4-methoxyphenyl)-2-pyridone (ZK 62771) enhanced the electrically evoked release of [³H]norepinephrine from superfused rat brain neocortex slices. 8-Bromo-cyclic GMP was without effect on the electrically evoked release. When [³H]norepinephrine release was enhanced by prolonging the electrical pulse duration from 2 msec to 10 msec, the relative inhibitory effect of the Ca²⁺ channel blocker Cd²⁺ and the relative facilitatory effect of the K⁺ channel blocker 4-aminopyridine remained unaffected. In striking contrast, the relative facilitatory effects of forskolin and 8-bromo-cyclic AMP were strongly reduced, whereas the effect of ZK 62771 was almost doubled.

When veratrine-induced release of [³H]norepinephrine from cortex synaptosomes was examined, the facilitatory effects of forskolin, 8-bromo-cyclic AMP, and ZK 62771 were even more pronounced than in brain slices. The data strongly support the hypothesis that a presynaptic adenylate cyclase system plays a facilitatory role in the stimulus-secretion coupling process in central noradrenergic nerve terminals.

Circumstantial evidence suggests a role for cyclic AMP in neurotransmitter release. Thus, in the peripheral nervous system it has been demonstrated that drugs thought to enhance intracellular cyclic AMP levels facilitate norepinephrine release *in vitro* (Wooten et al., 1973; Cubeddu et al., 1975; Pelayo et al., 1978; Stjärne et al., 1979). Similar data have been published concerning the release of norepinephrine from pheochromocytoma (PC12) cells (Rabe and McGee, 1983) and that of acetylcholine from bullfrog sympathetic ganglia (Kuba et al., 1981) and *Aplysia* neurons (Kandell and Schwartz, 1982).

However, in the mammalian central nervous system, evidence for the involvement of cyclic AMP in neurotransmitter release is less unambiguous. With respect to the release of catecholamines, for instance, Westfall et al. (1976) reported that dibutyryl cyclic AMP enhanced the electrically evoked release of [³H]dopamine from rat striatal slices. In contrast, Patrick and Barchas (1976) and Reisine et al. (1982) did not observe any effect of this membrane-permeating

cyclic AMP analogue on veratridine-induced and spontaneous release of dopamine, respectively, although it did enhance dopamine biosynthesis. Considering norepinephrine release, however, we and others have shown recently that membrane-permeating cyclic AMP analogues as well as the adenylate cyclase activator forskolin enhanced the depolarization-induced release of [³H]norepinephrine from rat neocortical slices (Wemer et al., 1982; Schoffmeier and Mulder, 1983a, b; Markstein et al., 1984). Unfortunately, data obtained with phosphodiesterase inhibitors are not fully in line with a proposed role for cyclic AMP in norepinephrine release, since the electrically evoked release from neocortical slices was enhanced by these drugs (Schoffmeier and Mulder, 1983a, b; Markstein et al., 1984), whereas K⁺-induced [³H]norepinephrine release was reduced (Dismukes and Mulder, 1976; Wemer et al., 1982).

Thus, at present the role of cyclic AMP in catecholamine release in the central nervous system is still unsettled. The differences between the various stimuli used to induce neurotransmitter release *in vitro* may well have contributed to conflicting data in the literature. Furthermore, it should be emphasized that direct evidence for the localization of cyclic AMP-generating systems in nerve terminals is lacking thus far and, consequently, the effects described above may well be indirect, i.e., not directly at the level of catecholaminergic nerve terminals. Therefore, in the present study we further investigated the role of this nucleotide in catecholamine release, focusing on the release of [³H]norepinephrine from rat neocortical slices and synaptosomes under quasi-physiological conditions.

Materials and Methods

Preparation and superfusion of brain tissue. Male Wistar rats (140 to 180 gm body weight) were sacrificed by decapitation and the brains were rapidly removed. Neocortical slices (0.3 × 0.3 × 2 mm) were prepared, labeled, and superfused, essentially as described previously (Schoffmeier et al., 1981). In short, slices (100 mg fresh tissue weight) were incubated for 15 min in 5 ml of Krebs-Ringer bicarbonate medium (for composition, see Mulder et al., 1983) containing 0.05 μM [³H]norepinephrine, in a 95% O₂/5% CO₂ atmosphere. After labeling, the slices were transferred to each of 24 superfusion chambers (0.2-ml volume; 4 mg of tissue/chamber) and subsequently superfused (0.25 ml/min) with medium at 37°C. After 30 min of superfusion the superfusate was collected in 10-min samples. During superfusion Ca²⁺-dependent [³H]norepinephrine release was induced at *t* = 50 min by exposing the slices to biphasic electrical pulses (1 Hz, 12 mA) of 2 or 10 msec duration for 5 min. Synaptosomes from the neocortex were prepared, labeled, and superfused as previously described (De Langen et al., 1979). The synaptosomes were superfused with Ca²⁺-free medium and depolarized at *t* = 50 min with veratrine (1 μM) for 5 min in the presence of 1.2 mM Ca²⁺.

In all experiments desipramine (10 μM) was present in the media in order to prevent reuptake of released [³H]norepinephrine. Drugs were added to the superfusion medium 20 min before depolarization. At the end of the experiment the remaining radioactivity was extracted from the tissue with 0.1 N HCl. The radioactivity in superfusion fractions and tissue extracts was determined by liquid scintillation counting.

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Calculation of release data. The efflux of tritium during each 10-min collection period was expressed as the fraction of the tritium content of the tissue at the beginning of the respective collection period. In order to calculate depolarization-induced [^3H]norepinephrine release, the spontaneous efflux of radioactivity was subtracted from the total overflow of tritium during stimulation and the following 25 min. Then depolarization-induced release was expressed as a percentage of the tritium content of the slices at the start of the stimulation period. The statistical significance of differences was determined with the two-tailed Student's *t* test.

Radiochemicals and drugs. L-[^3H]Norepinephrine hydrochloride (28 Ci/mmol) was purchased from the Radiochemical Centre (Amersham). The following drugs were obtained commercially: 4-aminopyridine (Baker), forskolin (Calbiochem), and 8-bromo-cyclic AMP, dibutyl cyclic AMP, isobutylmethylxanthine (IBMX), and veratrine (Sigma). 4-(3-Cyclopentyl-4-methoxyphenyl)-2-pyridone (ZK 62771) and desipramine were kindly donated by Schering A. G. and Ciba-Geigy, respectively.

Results

Effect of increasing intracellular cyclic AMP levels on the electrically evoked release of [^3H]norepinephrine from cortex slices. When slices, prelabeled with [^3H]norepinephrine, were superfused and stimulated electrically (1 Hz, 12 mA, 2-msec pulses) for 5 min, tritium release in excess of spontaneous efflux amounted to $4.30 \pm 0.05\%$ ($n = 72$) of total tissue content. The rate of spontaneous efflux of tritium during the 10-min period preceding electrical stimulation was $0.281 \pm 0.002\%$ min^{-1} of total tissue tritium. Forskolin, 8-bromo-cyclic AMP, dibutyl-cyclic AMP, and the phosphodiesterase inhibitors ZK 62771 and IBMX enhanced this release in a dose-dependent manner (Fig. 1). At the highest concentrations studied, forskolin (30 μM) and the cyclic AMP analogues (1 mM) also significantly enhanced (by about 20%) spontaneous tritium efflux. Similar effects were observed with the phosphodiesterase inhibitors at concentrations above 100 μM (data not shown). Therefore, these concentrations were not used in subsequent experiments.

Prolonging the electrical pulse duration from 2 msec to 10 msec enhanced the electrically evoked release to $9.21 \pm 0.06\%$ ($n = 16$) of total tissue content. Table I shows that Cd^{2+} (30 μM) reduced [^3H]norepinephrine release to about 30%, whereas 4-aminopyridine (100 μM) enhanced release to about 200% of control values, irrespective of the pulse duration. In contrast, the effects of forskolin (10 μM) and 8-bromo-cyclic AMP (300 μM) at 10 msec pulse duration were almost half those obtained at 2 msec pulse duration, whereas the effect of ZK 62771 (30 μM) was almost doubled.

Effect of increasing intracellular cyclic AMP levels on veratrine-induced release of [^3H]norepinephrine from cortex synaptosomes. When cortex synaptosomes were exposed to 1 μM veratrine for 5 min in the presence of 1.2 mM Ca^{2+} , the percentage of tritium released in excess of spontaneous tritium efflux was similar to that obtained with electrically stimulated brain slices (at 2 msec pulse duration), i.e., $5.85 \pm 0.21\%$ of total tissue content ($n = 16$). In this case the rate of spontaneous tritium efflux during the 10-min interval preceding depolarization was $0.362 \pm 0.008\%$ min^{-1} of total tissue tritium. As shown in Figure 2, forskolin (10 μM), 8-bromo-cyclic AMP (300 μM), and ZK 62771 (30 μM) facilitated synaptosomal [^3H]norepinephrine release, increasing it to about 160%, 220%, and 130% of control values, respectively.

Discussion

The electrically evoked release of tritium in excess of spontaneous efflux from superfused rat brain cortex slices prelabeled with [^3H]norepinephrine has been shown previously to consist of more than 80% of the unmetabolized amine and to be completely dependent on the presence of extracellular Ca^{2+} (Schoffemeer et al., 1981). Moreover, under our experimental conditions the electrically evoked release is abolished by the Ca^{2+} channel blocker Cd^{2+} and the Na^{+} channel blocker tetrodotoxin, whereas it is strongly en-

Figure 1. Effect of increasing intracellular cyclic AMP levels on the electrically evoked release of [^3H]norepinephrine from rat neocortex slices. Cortex slices prelabeled with [^3H]norepinephrine were superfused and stimulated electrically (1 Hz, 12 mA, 2-msec pulses) at $t = 50$ min for 5 min. Drugs were added to the superfusion medium 20 min before stimulation, except desipramine (10 μM) which was present throughout superfusion. Data represent means $\pm$ SEM. Each concentration was tested two to three times in quadruplicate. $\star$, $p < 0.01$ compared to control values (Student's *t* test).

TABLE 1

Effects of modulating the Ca^{2+} influx or increasing the intracellular cyclic AMP levels on $[^3\text{H}]$ norepinephrine release at different pulse durations

Cortex slices prelabeled with $[^3\text{H}]$ norepinephrine were superfused and stimulated electrically (1 Hz, 12 mA) at $t = 50$ min for 5 min with biphasic block pulses of either 2 msec or 10 msec duration. Drugs were added to the superfusion medium 20 min before stimulation except desipramine (10 μM), which was present throughout superfusion. Control $[^3\text{H}]$ norepinephrine release amounted to $4.15 \pm 0.08\%$ and $9.21 \pm 0.16\%$ of total tissue tritium at 2 msec and 10 msec pulse duration, respectively. Values represent means $\pm$ SEM of four experiments performed in quadruplicate.

Drugs	$[^3\text{H}]$ Norepinephrine Release (% of control)	
	2-msec pulses	10-msec pulses
None	100 ± 1.9	100 ± 1.7
Cd^{2+} (30 μM)	29.6 ± 6.2	28.3 ± 3.3
4-Aminopyridine (100 μM)	206 ± 14.7	214 ± 7.6
Forskolin (10 μM)	145 ± 2.9	126 ± 2.3^a
8-Bromo-cyclic AMP (300 μM)	158 ± 5.5	130 ± 3.1^a
ZK 62771 (30 μM)	118 ± 2.4	134 ± 2.1^a

^a $p < 0.01$ as compared to effects at 2 msec pulse duration (Student's t test).

hanced by the K^+ channel blocker 4-aminopyridine, as may be expected for action potential-induced release of the neurotransmitter from noradrenergic nerve terminals (Schoffeleer and Mulder, 1983b). When the release of $[^3\text{H}]$ norepinephrine from superfused synaptosomes is investigated as in the present study, electrical field stimulation is not feasible and activation of Na^+ channels, e.g., with veratrine, appears to be the stimulus which most closely resembles the physiological situation, in which the nerve terminal is depolarized upon invasion of an action potential (Blaustein, 1979).

The present study, using these quasi-physiological stimuli, lends further support to the hypothesis that cyclic AMP plays a facilitatory role in neurotransmitter release from central noradrenergic neurons. Forskolin, a diterpene that activates adenylate cyclase in rat brain slices, probably through a direct action on the catalytic unit of the enzyme (Seamon et al., 1981; Daly et al., 1982), was the most effective drug in facilitating the electrically evoked release of $[^3\text{H}]$ norepinephrine from cortex slices, being active in micromolar concentrations. At higher concentrations (0.01 to 1 mM) the membrane-permeating derivatives of cyclic AMP, dibutyl cyclic AMP and 8-bromo-cyclic AMP, also enhanced in a dose-dependent manner electrically evoked $[^3\text{H}]$ norepinephrine release as shown previously for veratrine-induced release (Schoffeleer and Mulder, 1983a). The former cyclic AMP analogue was less potent than the latter in this respect, probably due to the relatively slow dissociation of butyryl groups in cells (Heersche et al., 1971). Interestingly, as far as the involvement of cyclic nucleotides is concerned, this release-facilitating effect appeared to be specific for cyclic AMP in rat neocortex slices, since in concentrations up to 1 mM 8-bromo-cyclic GMP was without effect.

So far, the data obtained with forskolin and the cyclic AMP analogues are compatible with the hypothesis that the increase in depolarization-induced norepinephrine release is a consequence of increased cyclic AMP levels within noradrenergic nerve terminals. However, these data have no conclusive force with regard to the important question whether the cyclic nucleotide normally plays a facilitatory role in the release process triggered by depolarization and whether this involves a presynaptically localized adenylate cyclase. It has been reported previously that exposure of brain slices to depolarizing stimuli is somehow associated with enhanced intracellular levels of cyclic AMP (Shimizu et al., 1973; Zanella and Rall, 1973). Our data suggest that this effect of depolarization may indeed occur in central noradrenergic nerve terminals, since drugs increasing the intracellular cyclic AMP concentration through inhibition of cyclic AMP-phosphodiesterase, such as ZK 62771 (Schwabe et al.,

1976) and IBMX (Smellie et al., 1979), significantly enhanced the electrically evoked release of $[^3\text{H}]$ norepinephrine. Unexpectedly, these enhancing effects were relatively small compared to those of forskolin and the cyclic AMP analogues. However, prolonging the electrical pulse duration from 2 msec to 10 msec almost doubled the effect of ZK 62771, whereas the facilitatory effects of forskolin and 8-bromo-cyclic AMP were reduced. This would be a logical consequence of increased neuronal endogenous cyclic AMP levels at 10 msec pulse duration. Still, it might be argued that the reduced effects of forskolin and 8-bromo-cyclic AMP are due to a saturation of the release mechanism with Ca^{2+} at 10 msec pulse duration. However, this is unlikely in view of the data obtained from experiments examining the effects of Cd^{2+} and 4-aminopyridine on $[^3\text{H}]$ norepinephrine release evoked at either 2 msec or 10 msec pulse duration. If at 10 msec pulse duration saturation of the release mechanism with Ca^{2+} had occurred, the (relative) inhibiting effect of a blockade of voltage-sensitive Ca^{2+} channels with Cd^{2+} (Kostyuk, 1980; Schoffeleer and Mulder, 1983b) as well as the (relative) enhancing effect of their activation with 4-aminopyridine (Rogawski and Barker, 1983; Schoffeleer and Mulder, 1983b) would have been expected to be smaller than at 2 msec pulse duration. In contrast, the effects of these drugs were found to be independent of the pulse duration.

The data obtained with synaptosomes virtually exclude the possibility that the facilitatory effects described above are mediated indirectly, i.e., trans-synaptically or via interneurons, since in this preparation indirect effects on $[^3\text{H}]$ norepinephrine release are very unlikely (Mulder, 1982). First, homogenization of brain tissue, although preserving a major part of the nerve endings in the form of synaptosomes, destroys the ordered cellular structure and organization of the tissue, thereby largely eliminating interactions that might

Figure 2. Effect of increasing intracellular cyclic AMP levels on veratrine-induced release of $[^3\text{H}]$ norepinephrine from rat neocortex synaptosomes. Synaptosomes prelabeled with $[^3\text{H}]$ norepinephrine were superfused with Ca^{2+} -free medium and exposed to 1 μM veratrine at $t = 50$ min for 5 min in the presence of 1.2 mM Ca^{2+} . Drugs were added to the medium 20 min before depolarization, except desipramine (10 μM) which was present throughout superfusion. Data represent means $\pm$ SEM of four experiments in quadruplicate. $\star$, $p < 0.01$ compared to control release (Student's t test).

normally occur between closely apposed neurons. Second, neurotransmitters and other substances released upon depolarization are more efficiently removed from their sites of release by superfusion in synaptosomal preparations than in slices. Even in small slices part of the substances released has to travel some distance by diffusion through extracellular spaces within the slices before being removed by superfusion. In this situation of delayed removal, release of a neurotransmitter may result (for some time) in concentrations high enough to (partially) activate its receptors. This possibility is exemplified by the effects of α_2 -adrenoceptor antagonists on K^+ -induced [3H]norepinephrine release from brain slices or synaptosomes. Whereas agonists for (presynaptic) α_2 -adrenoceptors inhibit [3H]norepinephrine release in both preparations, antagonists enhance release in slices (Wemer et al., 1979) but not in synaptosomes (De Langen et al., 1979).

In the present study we were able to show that forskolin, 8-bromo-cyclic AMP, and ZK 62771 facilitated veratrine-induced [3H]norepinephrine release from cortex synaptosomes and these effects were even more pronounced than those on release from electrically stimulated slices. As a matter of fact, the facilitatory effect of phosphodiesterase inhibition by ZK 62771 as well as that of forskolin on synaptosomal norepinephrine release implies the presence of an adenylate cyclase system in noradrenergic nerve terminals.

In conclusion, the present study supports the view that cyclic AMP, produced within noradrenergic nerve terminals, plays a facilitatory role in depolarization-induced norepinephrine release in the central nervous system. The release-inhibiting effects of phosphodiesterase inhibitors at concentrations above 30 μM observed under certain experimental conditions, i.e., in K^+ -stimulated brain slices (Dismukes and Mulder, 1976; Wemer et al., 1982), may well be explained by a nonspecific action of these drugs. For example, if (some of) these drugs possess local anesthetic properties (see Schoffemeer and Mulder, 1983a), this will probably have less pronounced consequences at the more intense membrane depolarization caused by electrical field stimulation (see Schoffemeer et al., 1981).

The mechanism by which cyclic AMP facilitates the exocytotic process in noradrenergic varicosities remains obscure. The effect of the nucleotide may involve various processes acting in parallel or synergistically. For instance, cyclic AMP is thought to mediate phosphorylation of voltage-sensitive Ca^{2+} channels, thereby enhancing their conductance (Sulakhe and St. Louis, 1980; Reuter, 1983), and of several intracellular protein components which are thought to be critically involved in the stimulus-secretion coupling process (Nestler and Greengard, 1983). Moreover, cyclic AMP-dependent phospholipase A_2 may play an essential role in the exocytotic mechanism (Moskowitz et al., 1983, 1984).

Whether an increase in cyclic AMP levels in noradrenergic nerve terminals is an essential step in the exocytotic process (Rasmussen, 1980) or merely promotes the basic release mechanism (Rabe and McGee, 1983) cannot be derived from the present study and clearly requires further investigation.

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