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Enhanced Release of Dopamine-β-Hydroxylase from Sympathetic Nerves by Calcium and Phenoxybenzamine and Its Reversal by Prostaglandins

(norepinephrine/hypogastric nerves/vas deferens/guinea pigs)

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ABSTRACT **Dopamine-β-hydroxylase (EC 1.14.2.1), an enzyme localized in sympathetic synaptic vesicles, was released together with norepinephrine during *in vitro* stimulation of the hypogastric nerve that innervates the vas deferens of the guinea pig. Stimulation of the nerve in the presence of high concentrations of calcium (7.5 mM) or phenoxybenzamine caused a marked increase in the amounts of the enzyme and norepinephrine released into the bath. The augmentation of release of dopamine-β-hydroxylase by 7.5 mM calcium or phenoxybenzamine was reversed by prostaglandin E_2. These findings suggest that the release of the sympathetic neurotransmitter, norepinephrine, occurs by a process of exocytosis in which the vesicular content of soluble dopamine-β-hydroxylase is also released. The depolarization-induced exocytosis, which is stimulated by calcium, may be affected by prostaglandin E_2 or phenoxybenzamine through inhibition or enhancement of the actions of calcium in the release process.**

Release of epinephrine from the adrenal medulla in response to splanchnic-nerve stimulation or to acetylcholine is accompanied by release of ATP (1), soluble proteins† (2), and dopamine-β-hydroxylase (DOHase) that are stored in the vesicles of adrenergic neurons (3). These findings, together with electron microscopic evidence (4), have suggested that the secretion of the adrenal medulla occurs by a process of exocytosis in which the storage vesicles open at the cell membrane and release their contents into the extracellular space. DOHase, the enzyme that converts dopamine to norepinephrine, is highly localized in synaptic vesicles (5) and is released simultaneously with norepinephrine into the perfusate of isolated organs during electrical stimulation of sympathetic nerves‡ (6–7). This has been taken as evidence that sympathetic neurosecretion might occur by a similar process of exocytosis.

By the use of a sensitive assay for measuring DOHase activity (8, 9), we have investigated the *in vitro* release of the enzyme during stimulation of the hypogastric nerve to the vas deferens of the guinea pig. The effects of calcium, which is required for both release of epinephrine from the adrenal gland (10) and norepinephrine from sympathetic nerves (11, 12), and phenoxybenzamine, an α-blocking adrenergic agent that also causes a marked increase in the outflow of norepinephrine during nerve stimulation (13–16), have been studied. Since prostaglandin E_2 (PGE_2) blocks the enhanced outflow of norepinephrine from perfused organs caused by either high concentrations of calcium (17) or phenoxybenzamine (18), its effect on release of DOHase was also examined.

Abbreviations: DOHase, dopamine-β-hydroxylase; PG, prostaglandins.

† Banks, P., and K. Helle, *Biochem. J.*, **97**, 40c (1965).

‡ Geffin, L. B., B. G. Livett, and R. A. Rush, *J. Physiol.*, **204**, 58P (1969).

MATERIALS AND METHODS

Preparation of organs

Male albino guinea pigs, weighing 500–800 g, were killed by a blow on the head and exsanguinated. The vasa deferentia with attached hypogastric nerves were excised and put into 10-ml organ baths (19) at 37°C, containing medium of the following composition per liter. NaCl, 8.06 g; KCl, 0.35 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 294 mg; KH_2PO_4, 162 mg; glucose, 2.07 g, adjusted to pH 7.4 and aerated with 5% CO_2 in 95% O_2. The bath fluid was changed 4 times and then replaced by fresh medium containing 0.25% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). This was replaced after 10 min with 5.0 ml of albumin-containing medium, and the preparations were allowed to equilibrate for 5 min before the stimulation period was begun. The hypogastric nerves were stimulated electrically (5–7 V, 25 Hz, 5 msec) for 30 sec each minute for 30 min.

Assay of dopamine-β-hydroxylase

After the stimulation period the bath fluid was collected in (Teflon) plastic tubes kept on ice. A 0.2-ml aliquot was taken for the estimation of DOHase activity with tyramine as substrate (8, 9). Release of DOHase into the incubation medium was accompanied by the accumulation of an inhibitor of the enzyme that was overcome by the addition of $CuSO_4$ to the enzyme assay mixture. Maximal DOHase activity was obtained with a concentration of 13 μM $CuSO_4$, which was used in all subsequent determinations. Purified DOHase from bovine adrenal glands was found to be labile in the medium at 37°C, under the same conditions, unless 0.25% bovine serum albumin was added. Since the rate of the DOHase reaction was linear with time for 2.5 hr, incubations were continued for 2 hr for the first step (DOHase portion of the reaction), and 30 min for the second step (the phenylethanolamine-*N*-methyltransferase portion of the reaction).

Assay of norepinephrine

4.0-ml aliquots of the bath fluid were made acidic by the addition of 4.0 ml of 0.8 N perchloric acid and stored frozen.

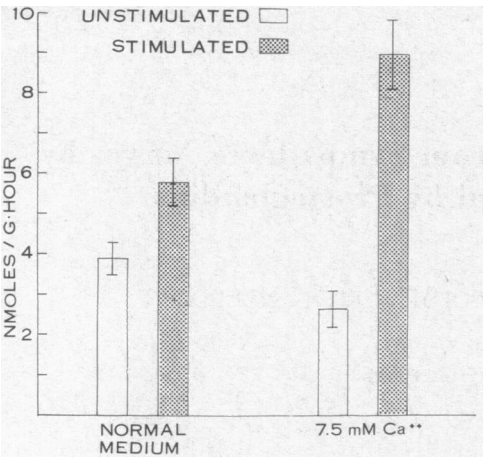

FIG. 1. Effects of hypogastric nerve stimulation and 7.5 mM calcium on DOHase activity released by guinea-pig vasa deferentia into the bath fluid during 30 min of stimulation. Results are expressed as nanomoles of octopamine formed from tyramine per gram of tissue per hour. Each bar represents the mean (±SE) for 10 preparations. $P < .02$, for stimulated versus unstimulated in normal medium. $P < .01$, for stimulated in medium containing 7.5 mM calcium versus stimulated in normal medium.

Samples were thawed, then centrifuged at 10,000 × *g* for 10 min. 10 mg of $Na_2S_2O_5$ and 10.0 ml of 2% NaEDTA were added to each sample before the norepinephrine was adsorbed on alumina columns and assayed fluorimetrically by the trihydroxyindole method (20).

Drugs

Phenoxybenzamine·HCl was kindly supplied by Smith, Kline, and French Lab., Philadelphia, Pa. Prostaglandins E_1 and E_2 were gifts of Dr. John Pike, The Upjohn Co., Kalamazoo, Mich.

RESULTS

Effect of calcium on release of dopamine-β-hydroxylase

Although DOHase activity was already present in the medium with unstimulated organs, electrical stimulation of the hypogastric nerve caused about a 50% increase in release of the enzyme (Fig. 1). Stimulation in the presence of relatively high calcium concentrations (7.5 mM) resulted in a large increase in the release of the enzyme, to concentrations more than double those found in unstimulated preparations. There was no significant change in the concentration of enzyme found in the incubation medium of unstimulated preparations incubated in 7.5 mM calcium.

Effect of prostaglandins E_1 and E_2 on release of dopamine-β-hydroxylase in a medium with 7.5 mM calcium

The augmented release of DOHase during stimulation in a medium with 7.5 mM calcium was reversed by PGE_2 to a level comparable to that normally produced by nerve stimulation (Fig. 2). PGE_1, at the same molar concentrations (1.8×10^{-6} M), was less effective than PGE_2, causing only a partial inhibition of the enhanced release of the enzyme in 7.5 mM calcium. Neither PGE_1 nor PGE_2 had any significant effect on the release of the enzyme by stimulated or unstimulated organs in medium containing a normal concentration of calcium (2.04 mM).

Effect of phenoxybenzamine on release of dopamine-β-hydroxylase

Phenoxybenzamine (3.0×10^{-5} M) caused a marked increase in release of DOHase during nerve stimulation, to levels comparable to those found in a medium with 7.5 mM calcium (Fig. 3). There was no difference in the amount of the enzyme recovered in the medium of unstimulated preparations incubated in the presence of phenoxybenzamine. PGE_2 (1.8×10^{-6} M, about $^1/_{16}$ the molar concentration of phenoxybenzamine) caused a decrease in the release of

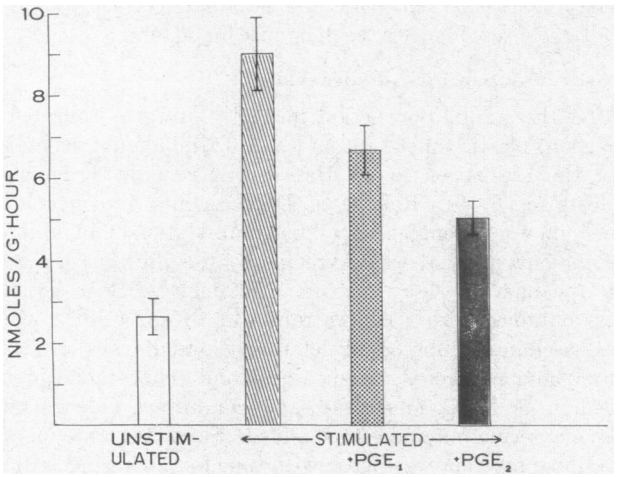

FIG. 2. Effects of PGE_1 and PGE_2 on DOHase activity released by guinea-pig vasa deferentia into the incubation medium during 30 min of stimulation of the hypogastric nerves in medium containing 7.5 mM calcium. Results are expressed as nanomoles of octopamine formed from tyramine per gram of vas deferens per hour. Each bar represents the mean (±SE) for 10 preparations. $P < .01$, PGE_2 versus 7.5 mM calcium alone.

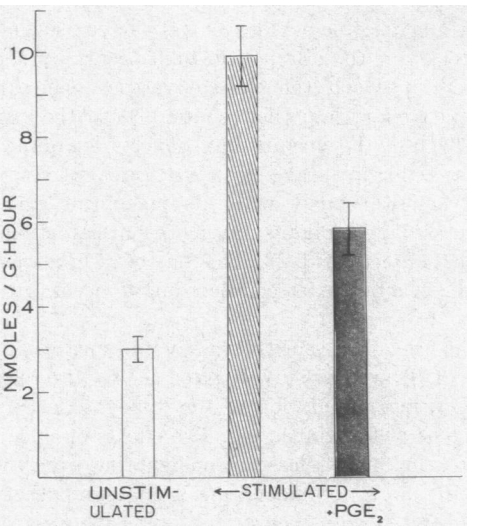

FIG. 3. Effects of phenoxybenzamine and PGE_2 on DOHase activity released into the incubation medium by guinea-pig vasa deferentia during 30-min of stimulation of the hypogastric nerve. Results are expressed as nanomoles of octopamine formed from tyramine per gram of vas deferens per hour. Each bar represents the mean (±SE) for 10 preparations. All 3 groups differ significantly from each other ($P < .01$).

DOHase in preparations treated with phenoxybenzamine to the normal level found during nerve stimulation (Fig. 3).

Effect of phenoxybenzamine on release of norepinephrine

While no detectable norepinephrine (less than 10 ng) was found in the incubation medium after nerve stimulation in the absence of drugs, phenoxybenzamine caused a large increase in the concentration of norepinephrine in the medium (Table 1). Like its effect on the release of DOHase, PGE inhibited the increased release of catecholamines produced by phenoxybenzamine.

DISCUSSION

Evidence has been presented showing that release of norepinephrine occurs by a process that includes release of DOHase. This enzyme is a large molecule located in the storage vesicles of adrenergic neurons‡ (5, 21). In addition, the ratio of norepinephrine to DOHase released into the medium is relatively constant and similar to the ratio of norepinephrine to soluble DOHase found in organ homogenates.§ It appears, therefore, that sympathetic neurosecretion involves exocytosis, during which a comparatively large opening is formed between the cavity of the nerve vesicles and the synaptic cleft. The results presented here suggest that exocytosis in the sympathetic nerve terminals, like that in the adrenomedullary cells (10) is a calcium-dependent process and elevated concentrations of calcium are associated with increased release of the vesicular contents into the synaptic cleft (Fig. 1). Absence of calcium also prevents the release of DOHase from the perfused bovine spleen during electrical stimulation of the splenic nerve (6). The antagonistic action of PGE_2 and PGE_1 on the increased release of DOHase in the presence of increased calcium is similar to their inhibition of the effect of 5 mM calcium on norepinephrine outflow in the perfused cat-spleen (17). This indicates that prostaglandins interfere with the action of calcium and, thus, may play a regulatory role in the calcium-dependent secretion of norepinephrine and DOHase.

The increase in outflow of norepinephrine during sympathetic nerve stimulation caused by phenoxybenzamine (13–16) has been regarded as a consequence of the inhibition of norepinephrine reuptake (14, 22). However, cocaine and desmethylimipramine, compounds that inhibit neuronal uptake of norepinephrine just as effectively as phenoxybenzamine (22, 23), cause a much smaller increment in efflux of norepinephrine during nerve stimulation (15). The α-receptor that blocks the action of phenoxybenzamine does not appear to account for the increase in outflow of norepinephrine, since this alone causes only a slight increase in release of norepinephrine from the spleen during nerve stimulation (15). The enhance drelease of DOHase during stimulation in the presence of phenoxybenzamine (Fig. 3) suggests that the increased outflow of norepinephrine may be due to an increase in transmitter release (13–15), which is coupled with extrusion of increased amounts of soluble DOHase. The increased release of the enzyme by phenoxybenzamine only when the nerve is stimulated suggests that it permits the nerve to remain in a conformational state that allows large molecules to be secreted. The reversal of this effect by PGE_2 could reflect a competition between phenoxybenzamine and PGE_2 for a prejunctional site that regulates the release of neurotransmitter (17). Phenoxybenzamine has been known to inhibit release of endogenous PGE_2 from the perfused cat spleen during sympathetic nerve stimulation (24). Thus, phenoxybenzamine may increase neurosecretion of norepinephrine and DOHase by interfering with a regulatory inhibition by endogenous prostaglandin. A normal rate of release would then be restored upon addition of exogenous PGE_2.

The role of calcium in the stimulus–secretion coupling by which both adrenal medullary cells and sympathetic nerves release catecholamines and vesicular proteins remains unknown (25). Like the process of excitation–contraction coupling in muscle, neuronal depolarization results in an increased influx of calcium that is required for secretion of neurotransmitter. Unlike the secretion of the adrenal medulla that is not affected by PGE_2 (26, 27), coupled release of norepinephrine and DOHase from sympathetic nerves may be regulated by endogenous prostaglandins by their control of either the intracellular accumulation of calcium or its subsequent function in the process of neurosecretion (26).

Both calcium and phenoxybenzamine cause an enhanced release of norepinephrine and the vesicular protein, DOHase. PGE_2 blocks the releasing action of calcium and phenoxybenzamine. Thus, phenoxybenzamine may enhance the entry of calcium into the neuron or slow its efflux. This effect of phenoxybenzamine might be exerted directly at the neuronal membrane or indirectly, through its inhibitory action on the release of prostaglandins.

TABLE 1. *Effects of phenoxybenzamine and prostaglandins E_2 on release of norepinephrine into the incubation medium during stimulation of the hypogastric nerve to the vas deferens*

Treatment	Norepinephrine released (ng/g tissue)
Unstimulated	40 ± 5
Stimulated	487 ± 77
Stimulated + PGE_2	218 ± 33

Guinea-pig vasa deferentia were incubated for 30 min *in vitro* in the presence of phenoxybenzamine (3×10^{-5} M), or phenoxybenzamine and PGE_2 (1.8×10^{-6} M). The hypogastric nerve was stimulated (5–7 V, 25 Hz, 5 msec) for 30 sec each min. Results are expressed as the mean (±SE) norepinephrine recovered in each bath per gram of tissue incubated for groups of 10 preparations.

$P < .02$, unstimulated versus stimulated in the presence of phenoxybenzamine and PGE_2. $P < .01$, phenoxybenzamine-treated versus phenoxybenzamine and PGE_2-treated preparations.

R. W. was a research associate in the Pharmacology–Toxicology Program of the National Institute of General Medical Sciences, N.I.H.

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