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THE EFFECT OF PRENYLAMINE AND ORGANIC NITRATES ON THE BIOENERGETICS OF BOVINE CATECHOLAMINE STORAGE VESICLES

MARTIN GRØNBERG, OLE TERLAND, EYSTEIN S. HUSEBYE and TORGEIR FLATMARK*
Department of Biochemistry, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway

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Abstract—We have compared the cardioprotective agents prenylamine and glyceryl trinitrate (GTN) with respect to their effects on the bioenergetics of catecholamine storage vesicles. Chromaffin granule ghosts, which have a well preserved ability to actively transport and store catecholamines, were used as a model for adrenergic synaptic vesicles due to their functional similarity. Prenylamine, which partially and reversibly deplete the endogenous stores of noradrenaline in adrenergic nerves and ganglia, was found to inhibit the generation of the transmembrane proton electrochemical gradient driven by a H^+-ATPase, mainly by acting as an uncoupler of this ATPase. The inhibition of the energy dependent dopamine uptake (and noradrenaline biosynthesis) by prenylamine could be accounted for by its effect on the bioenergetics of the storage vesicles. The organic nitrates glyceryl trinitrate and isosorbide dinitrate also partly inhibited the catecholamine uptake in parallel with their effects on the proton electrochemical gradient. It is concluded that GTN is a weak catecholamine depletor. Experiments with 3-morpholinosydnonimin-hydrochloride, a source of nitric oxide (NO), opens up the possibility that the mechanism of inhibition of the bioenergetics of chromaffin granule ghosts by GTN is mediated by NO.

A reduction in the level of endogenous myocardial catecholamines by chemical [1, 2] or surgical [1] means, has been shown to reduce the resulting cell damage in experimental ischemia [1, 2]. Evidence is gathering that this mechanism may be of clinical importance. Thus, the calcium antagonist prenylamine is believed to have additional cardioprotective effects *in vivo* [3] because it partially and reversibly depletes the noradrenaline stores in adrenergic nerves and ganglia [4, 5] and in isolated rat heart preparations [3, 5]. This depletion is the result of an inhibition of catecholamine uptake into the storage vesicles of sympathetic tissue, and a release of catecholamines [4, 5], but the precise mechanism responsible for these effects is unknown.

It has been suggested that the organic nitrates, in particular glyceryl trinitrate (GTN†), like prenylamine, have important cardioprotective effects [6, 7]. The mechanism of action responsible for these beneficial effects during long term administration to patients with angina pectoris [8] is, however, poorly understood. It was therefore of interest to see if the organic nitrates, like prenylamine, had any effects on the bioenergetics of catecholamine storage vesicles, and to describe in more detail the effects of prenylamine in this system.

The catecholamine storage vesicles of the adrenomedullary cell are structurally and functionally very similar to the storage vesicles of sympathetic neurons [9, 10] found in several organs including the central and peripheral nervous system and the heart [11]. Thus, the chromaffin cell serves as a representative model of the catecholaminergic neurons and noradrenergic nerve terminals.

MATERIALS AND METHODS

Chemicals. ATP (disodium salt, essentially vanadium free), acridine orange, ascorbate, prenylamine lactate, isosorbide dinitrate, and catalase were from the Sigma Chemical Co. (Poole, U.K.). Oxonol VI was from Molecular Probes (Eugene, OR). Glyceryl trinitrate (25 mg/mL, in ethanol) was from Hydro-Pharma (Oslo, Norway). Isosorbide-5-mononitrate was a gift from Pharmacia (Norway). SIN-1 was a gift from Dr E. Schraven (Cassella AG, Frankfurt, F.R.G.). All other chemicals were of reagent grade.

Preparation of chromaffin granule ghosts. Chromaffin granule ghosts were prepared from bovine adrenal glands as previously described [12, 13].

Assay of noradrenaline synthesis. The energy for the synthesis of noradrenaline in chromaffin granules is provided by the H^+-ATPase (EC 3.6.1.34) which generates a proton electrochemical gradient (interior acidic and positive) necessary to transport dopamine into the granules where it is converted to noradrenaline [14–17] by the action of dopamine β-monooxygenase (EC 1.14.17.1). Active transport of dopamine can thus be selectively determined by measuring the rate of noradrenaline synthesis [18]. All experiments were performed in the standard assay medium containing 10 mM Hepes (pH 6.5 with KOH), 0.3 M sucrose, 50 mM KCl, 2.5 mM ATP and 2.5 mM $MgSO_4$. The temperature was 37° in all experiments. When the intravesicular synthesis of noradrenaline from extravesicular dopamine was

* Correspondence to: Prof. Torgeir Flatmark.

† Abbreviations: acridine orange, (3,6-bis[dimethylamino]acridinum chloride hemi[zinc chloride]); GTN, glyceryl trinitate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonic acid; NA, noradrenaline; NO, nitric oxide; oxonol VI, bis[3-propyl-5-oxoisoxanol-4-yl]pentamethine oxonol; SIN-1,3-morpholino-sydnonimin-hydrochloride.

assayed, the medium was supplemented with 30 μM dopamine (as the hydrochloride), 5 mM ascorbate, and 0.1 mg/mL catalase and inhibitors as indicated. In order to measure selectively the net, energy-dependent uptake of dopamine, controls were performed in the absence of MgATP, and the amount of noradrenaline formed under these conditions was subtracted from the MgATP-stimulated noradrenaline formation [15, 18]. The reaction was initiated by the addition of chromaffin granule ghosts (0.5 mg/mL) and stopped by 1:1 dilution with ice-cold ethanol:acetic acid (90:10 v/v). The samples were centrifuged and the supernatants were assayed for noradrenaline by high performance liquid chromatography as in Ref. 19.

Assay of Mg^{2+}-ATPase activity and proton pump activity. ATPase activity was assayed in the standard assay medium with inhibitors as indicated. The inhibitors were preincubated with the chromaffin granule ghosts (0.05 mg/mL) for 5 min before the reaction was initiated by the addition of MgATP. The formation of ADP was measured by high performance liquid chromatography as in Ref. 17 or spectrophotometrically as in Ref. 20. The MgATP dependent generation of a transmembrane pH gradient or potential was followed by the spectroscopic probes acridine orange and oxonol VI, respectively [17, 20]. We have previously shown [17] that our preparations contain <5% mitochondrial F_1-ATPase activity and no detectable F_1F_0-ATPase activity (i.e. oligomycin-sensitive ATPase activity). Vanadate-sensitive ATPase activity accounts for 15–20% of the overall MgATPase activity, but is not involved in proton translocation [17] and will not interfere with the determination of the transmembrane pH gradient or potential. Thus the H^+-ATPase activity accounts for 75–80% of the overall MgATPase activity [17].

All data represent the mean of at least two experiments, and, unless otherwise stated, the standard deviation was always less than 5% of the control value.

Other analytical procedures. Dopamine β-monooxygenase was assayed as in Ref. 21. Phosphatidylinositol kinase (EC 2.7.1.67) activity was assayed as in Ref. 22. Protein was determined according to Bradford [23] with bovine serum albumin as the standard.

All experiments were performed in glass tubes. Care was taken to avoid adsorption of glyceryl trinitrate to plastic equipment [24].

RESULTS

The effects of prenylamine on the bioenergetics and dopamine uptake in chromaffin granule ghosts

The chromaffin granule ghosts synthesize noradrenaline at a net rate of about 1 nmol NA/min/mg at the selected experimental conditions, including 30 μM dopamine in the external medium (Fig. 1). In the absence of a pre-existing pH gradient, this process is dependent upon MgATP for the transport of dopamine across the granule membrane and was found to be inhibited by low concentrations of prenylamine (Fig. 1). From Fig. 1 it is also seen that the overall MgATPase activity was slightly stimulated by low concentrations (<20 μM) of prenylamine whereas higher concentrations were inhibitory (approx. 25% inhibition at 100 μM). Proton pump activity, measured as the generation of a pH gradient or the generation of a membrane potential (Fig. 1),

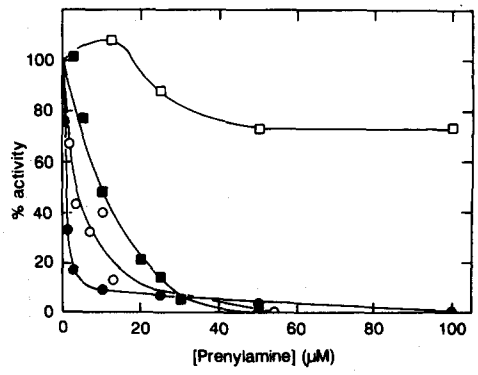

Fig. 1. The effects of prenylamine on the overall Mg^{2+}-ATPase activity of chromaffin granule ghosts (□), the rate of generation of a pH gradient determined by the acridine orange method (○), the generation of a membrane potential determined by the oxonol VI method (●), and the biosynthesis of noradrenaline from added dopamine (■). Uninhibited Mg^{2+}-ATPase activity was 240 nmol/min/mg and noradrenaline synthesis was 1 nmol NA/min/mg. Proton pump activity is expressed in arbitrary units. For experimental details, see text.

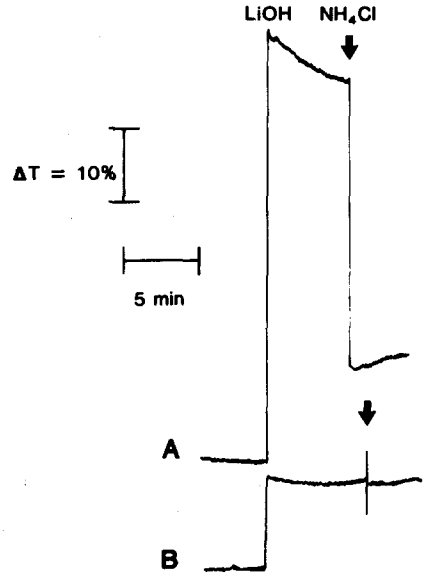

Fig. 2. The prevention by prenylamine of the generation of a pH gradient across the chromaffin granule ghost membrane. Chromaffin granule ghosts were incubated with acridine orange in the standard assay buffer adjusted to the internal pH of the ghosts. A pH gradient (inside acidic) of approx. 1 pH unit was generated by the addition of LiOH to the medium (trace A). The pH gradient was then dissipated by the addition of 1 mM NH_4Cl (arrows). When prenylamine (25 μM) was present in the medium before the addition of LiOH, the generation of a pH gradient was totally inhibited (trace B).

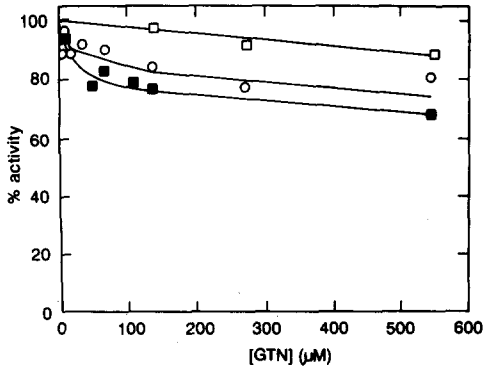

Fig. 3. The effects of glyceryl trinitrate on the overall Mg^{2+}-ATPase activity of chromaffin granule ghosts (□), proton pump activity (○) and the biosynthesis of noradrenaline from dopamine (■). Uninhibited Mg^{2+}-ATPase activity was 240 nmol/min/mg and noradrenaline synthesis was 1 nmol/min/mg. Proton pump activity is expressed in arbitrary units.

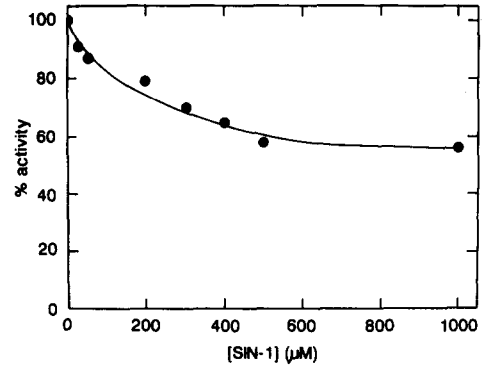

Fig. 4. The effect of SIN-1 on the proton pump activity of chromaffin granule ghosts. Proton pump activity was measured by the acridine orange method, and the activity expressed in arbitrary units.

Table 1. The effect of organic nitrates on the proton pump activity of chromaffin granule ghosts

Compound	Relative activity ± SD	N
No additions (control)	100 ± 4.9	6
Glyceryl trinitrate		
0.25 mM	89 ± 0.8	3
0.5 mM	74 ± 5.3	3
1.2 mM	52 ± 2.0	3
Isosorbide dinitrate		
1.0 mM	100 ± 4.0	6
1.2 mM	98 ± 4.7	4
2.5 mM	78 ± 1.4	3
Isosorbide 5-mononitrate		
1.2 mM	100 ± 3.7	4

Proton pump activity was measured by the acridine orange method, and the activity is expressed in arbitrary units.

was, however, inhibited in parallel with the noradrenaline synthesis, with complete inhibition at 55 and 100 μM of prenylamine, respectively. In the presence of 25 μM prenylamine no pH gradient could be imposed across the granule membrane by adding LiOH to the external medium in the absence of added MgATP (Fig. 2).

The effects of glyceryl trinitrate

The rate of synthesis of noradrenaline was inhibited by GTN (Fig. 3). GTN was found to inhibit the overall MgATPase activity (Fig. 3), as well as the proton pump activity as measured by the acridine orange method (Fig. 3 and Table 1) and by oxonol VI (data not shown). The degree of inhibition of the proton pump was not altered by including cysteine (5 mM), β-mercaptoethanol (11 mM) or dithiotreitol (1 mM) in the assay medium (data not shown).

The effects of isosorbide dinitrate and isosorbide-5-mononitrate

Isosorbide dinitrate revealed the same effects as GTN on the proton pump activity, but was less potent (Table 1). Isosorbide-5-mononitrate was virtually without any effect in this experimental system (Table 1).

The effect of SIN-1 on proton pumping

SIN-1, a source of nitric oxide which has been proposed as the mediator of the effects of GTN on vascular smooth muscle [25–27], inhibited the proton pump activity of chromaffin granule ghosts (Fig. 4).

Other investigations

Phosphatidylinositol kinase activity was assayed in chromaffin granule ghosts as it is inhibited by a number of lipophilic compounds which perturb the membrane structure [28], but it was not affected by glyceryl trinitrate. None of the compounds tested had any effect on dopamine β-monooxygenase activity.

DISCUSSION

The basal mechanism of prenylamine action on the bioenergetics of catecholamine storage vesicles

The effect of prenylamine on catecholamine metabolism is of considerable importance with respect to its cardioprotective and antianginal effects [3, 5]. We have used chromaffin granule ghosts as a model for adrenergic synaptic vesicles because the former are easily isolated in rather large quantities with a high purity from the bovine adrenal medulla [12], and they are functionally well preserved with respect to proton translocation and to catecholamine transport, synthesis (noradrenaline from dopamine) and storage [15–18]. We have found that prenylamine is a potent inhibitor of the bioenergetics of chromaffin granule ghosts. The effects of prenylamine on the overall MgATPase activity and proton pump activity is best explained assuming that the compound in low concentrations acts as an uncoupler of the H^+-ATPase (Fig. 1), whereas higher concentrations also inhibits the MgATP hydrolysis. From Fig. 2 it is evident that the reduced efficiency of the proton pump is due to an increased permeability for protons across the granule membrane

induced by prenylamine. As expected, the inhibition of noradrenaline synthesis closely parallels that of proton pumping and explains the ability of prenylamine to cause release of and to prevent uptake of catecholamines in synaptic vesicles [4, 5].

Glyceryltrinitrate and catecholamine metabolism

The mechanism of the beneficial effect of long term GTN administration to patients with angina pectoris [8] is poorly understood as the vascular tolerance observed during experimental conditions [29, 30] seems not to impair this effect [8]. We have looked for a possible explanation for this apparent discrepancy in the effects of GTN on catecholamine metabolisms. The net amount of catecholamines in adrenal storage granules is the result of a dynamic equilibrium between the rate of energy-dependent uptake from and energy-independent release to the cytoplasm. Even a small decline in the efficiency of the uptake mechanism may therefore cause a decline in the vesicular content of catecholamines. GTN was found to inhibit the biosynthesis of noradrenaline in chromaffin granule ghosts (Fig. 3), an effect which cannot be attributed to inhibition of dopamine β-monooxygenase. Although we have not analysed for a direct effect of GTN on the catecholamine carrier, the correlation between proton pump inhibition and the inhibition of dopamine transport, argues against such an effect. The inhibition of noradrenaline synthesis is therefore considered to be secondary to its effect on the bioenergetics of the chromaffin granules. Since even high concentrations of GTN were found to have no effect on the phosphatidylinositol kinase activity of chromaffin granules, it is unlikely that the inhibition of membrane bioenergetics by GTN is a result of an unspecific membrane perturbation [20, 28]. The observed inhibition of proton pumping by SIN-1 (Fig. 4) may be due to a direct effect of the compound on the proton pump. It should be noted that SIN-1 spontaneously liberates NO which has been proposed as the mediator of GTN induced relaxation of smooth muscle [25–27] and that the maximal inhibition obtained with SIN-1 is about 50% which is comparable to the inhibition obtained with 1.2 mM GTN (Table 1), the highest concentration tested.

The chromaffin granule H^+-ATPase is very sensitive to modification of its functional thiol groups [16, 17]. Thiol groups have been suggested to play a role in the formation of NO from GTN [27, 29], and it is interesting to note that neither cysteine, dithiotreitol nor β-mercaptoethanol protected the proton pump against inhibition by GTN. It has recently been shown that the H^+-ATPase contains a regulatory site which is modified by inorganic nitrate and which is accessible only from the inside of the vesicle [31]. It is possible that GTN, a lipid-soluble and membrane permeable molecule, may exert its inhibitory effect directly on the ATPase via this site.

Isosorbide dinitrate was less potent than GTN with respect to inhibition of proton translocation (Table 1) and noradrenaline synthesis. This was somewhat surprising as isosorbide dinitrate has been reported as an inhibitor of the H^+-ATPase of rat liver multivesicular bodies [32]. Isosorbide 5-mononitrate was without effect on proton translocation, and it may be significant that isosorbide 5-mononitrate is water soluble and not able to diffuse through the membrane and modify the H^+-ATPase from the inside of the vesicle [31].

Partial inhibition by GTN (approx. 20%) was observed at concentrations below 50 μM. This concentration range is orders of magnitudes higher than the estimated therapeutic plasma concentration of GTN (which is in the nanomolar range), but comparable to that previously used in *in vitro* studies considered to be of clinical significance [29, 30], notably on the activation of partially purified guanylate cyclase by GTN [29] and on the GTN-induced relaxation in tolerant and non-tolerant blood vessels [30]. Further, on long term treatment with GTN one has to consider the possibility that the drug accumulates in hydrophobic compartments like the membranes of cells and organelles. Part of the effect of long term GTN treatment in chronic ischemic heart disease may therefore be due to a modulation of sympathetic nervous activity secondary to a reduced ability to synthesize and store catecholamines, an effect known to be beneficial in experimental myocardial ischemia [1, 2].

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