Guanylate cyclase stimulation by nitro-compounds is dependent on free Ca^{2+} [1]

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Summary. The stimulatory effect of nitro-compounds on arterial and hepatic guanylate cyclase became significantly depressed at 0.2 μM and higher concentrations of free Ca^{2+}. The basal enzyme activity proved to be Ca^{2+}-independent.
Key words. Pig, carotid artery; artery, pig carotid; guanylate cyclase; nitro-compounds; Ca^{2+}, free.

Guanylate cyclase stimulation by nitro- and nitroso-compounds is suspected to be the underlying mechanism for their vasodilatory activity[2,3]. The effect is dependent on the presence of reducing agents such as thiols and on Mg^{2+} or Mn^{2+} as divalent cations. Since the activated enzyme uses Mg^{2+} more effectively than Mn^{2+}, Mg^{2+} may be a physiological cofactor[4]. On the other hand, a possible effect of Ca^{2+} on nitro-stimulated guanylate cyclase has not been fully elucidated as yet, since, in previous studies, Ca^{2+} concentrations in the millimolar range have been found necessary to influence the enzyme[5-8]. It is rather unlikely that such effects are of any physiological importance. In the present communication it is shown, by using an EGTA buffering system and calculating the free Ca^{2+} concentration, that free Ca^{2+}, at concentrations which are likely to be present intracellularly, markedly influences guanylate cyclase activation by nitro-compounds.

Materials and methods. Carotid arteries from freshly slaughtered pigs were freed from loose connective tissue and the intima-media layer stripped from the adventitial layer. The strips were minced, homogenized by means of an Ultra-Turrax at full speed for 3–5-sec intervals and once again homogenized using a tight-fitting glass pestle in 10 mM Hepes (pH 7.4), 0.25 M sucrose, 0.5 mM EGTA, and 1 mM mercaptoethanol. Following a 1000 × g centrifugation (10 min) the supernatant was further centrifuged at 100,000 × g for 60 min at 4 °C and the resulting supernatant was dialyzed for 4 h in the cold against 10 mM Hepes (pH 7.4), 2 mM EGTA, 1 mM mercaptoethanol, 0.01% activated charcoal, and stored under liquid nitrogen. Assays were performed within 1 week, during which time no loss in guanylate cyclase activity was observed. Protein was determined by dye-binding (Coomassie brilliant blue G-250) using the assay kit provided by Bio-Rad (Richmond, CA). Hepatic supernatant was obtained from rats, which were killed by a blow on the head and exsanguinated. The liver was rapidly perfused in situ (portal vein) with 20 ml ice-cold 10 mM Hepes (pH 7.4), 0.25 M sucrose, 0.5 mM EGTA, 1 mM mercaptoethanol, and then removed, minced, extensively washed with the above medium, and homogenized in a glass homogenizer with a teflon pestle (10 strokes, setting 7). The following steps including centrifugation and dialysis of a 100,000 × g supernatant were the same as described for the arterial supernatant.

Guanylate cyclase was determined in 120 μl of a reaction mixture containing 0.2 mM [α-^{32}P]GTP (0.1–0.5 μCi, NEN, Boston, MA), 0.2 mM cGMP (in order to 'trap' phosphodiesterase activity), 80 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES, pH 7.4), 2 mM EGTA, 2 mM dithiothreitol, 5 mM creatine phosphate, 5 U creatine kinase and, unless otherwise, indicated 5 mM $MgCl_2$. Incubation was started by adding 100–200 μg protein, carried out at 37 °C for 10 min, and terminated by addition of 60 mM EDTA (20 μl). Cyclic [^{32}P]GMP was quantitated as described by Birnbaumer et al.[9] by means of cyclic [^{3}H]GMP (NEN, Boston, MA) for recovery monitoring. The concentration of free Ca^{2+} in the guanylate cyclase incubation medium was calculated for each addition of $CaCl_2$ by a computer program developed by Piascik et al.[10] using a Digital PDP 11/10 computer.

Results and discussion. Figure 1 illustrates the Ca^{2+}-dependence of arterial guanylate cyclase stimulation induced by nitro-compounds. At 0.2 μM free Ca^{2+}, the stimulatory effects of 2', 3'-di-O-nitro-5'-(N-ethylcarboxamide)-adenosine[11] (dinitro-NECA, kindly supplied by Byk Gulden Lomberg Chem. Fa-

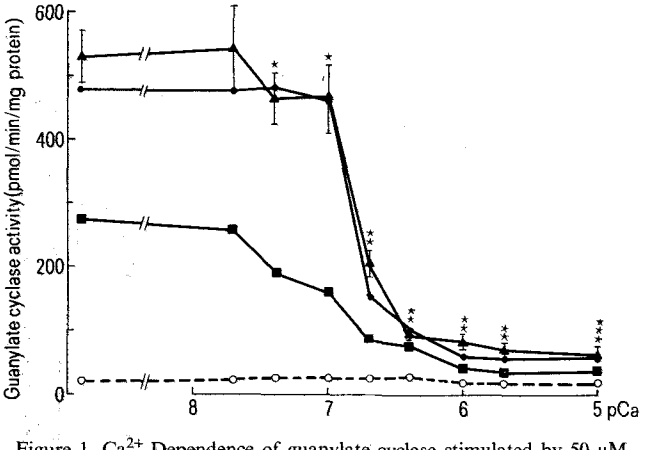

Figure 1. Ca^{2+}-Dependence of guanylate cyclase stimulated by 50 μM dinitro-NECA, sodium nitroprusside, and isosorbide dinitrate (enzyme substrate Mg-GTP) as well as under basal conditions (enzyme substrate Mn-GTP, ○——○) in a 100,000 × g supernatant obtained from hog carotid arteries. For dinitro-NECA (▲—▲) the means ± SEM (n = 5) are shown, for sodium nitroprusside (●—●) and isosorbide dinitrate (■—■) the means of triplicate determinations of a single experiment. Significance symbols: * 0.05 > p > 0.01; ** 0.01 > p > 0.001; *** p < 0.001.

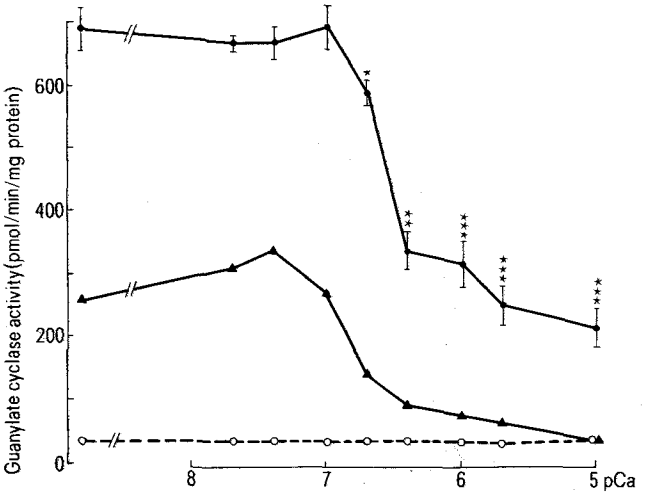

Figure 2. Ca^{2+}-Dependence of guanylate cyclase stimulated by 50 μM sodium nitroprusside and dinitro-NECA (enzyme substrate Mg-GTP) as well as under basal conditions (enzyme substrate Mn-GTP, ○——○) in a 100,000 × g rat liver supernatant. For sodium nitroprusside (●—●) the means ± SEM (n = 5) are shown, for dinitro-NECA (▲—▲) the means of triplicate determinations of a single experiment. For significance symbols see figure 1.

brik, Konstanz, FRG), sodium nitroprusside, and isosorbide dinitrate were markedly inhibited. Inhibition was almost maximal at 1 μM Ca^{2+}. Basal guanylate cyclase activity was unaffected over the Ca^{2+} concentration range of 0.02–10 μM, irrespective of whether Mn-GTP (fig. 1) or Mg-GTP served as the enzyme substrate. In the presence of Mn-GTP, basal guanylate cyclase activity was approximately 6-fold higher than in the presence of Mg-GTP.

In order to rule out that the observed effects of free Ca^{2+} on guanylate cyclase stimulation represent a unique property of arterial guanylate cyclase, similar experiments were carried out on a 100,000 × g supernatant obtained from rat liver. In this preparation, the dependence of guanylate cyclase stimulation by dinitro-NECA and sodium nitroprusside on Ca^{2+} was very similar to that observed in the arterial supernatant (fig. 2). Again, basal guanylate cyclase activity proved to be Ca^{2+} independent, irrespective of whether Mn-GTP or Mg-GTP served as the substrate.

Thus, guanylate cyclase stimulation by the nitro-compounds dinitro-NECA, sodium nitroprusside, and isosorbide dinitrate was strongly inhibited by free Ca^{2+} concentrations well within the physiological range, i.e., the half-maximal inhibitory concentration of Ca^{2+} was about 0.5 μM. Since the basal guanylate cyclase activity proved to be completely independent of Ca^{2+}, a direct interaction of Ca^{2+} and the nitro-compounds on the guanylate cyclase molecule may be assumed. However, the hypothesis is rather speculative at present due to the lack of experimental evidence for the putative nitro- or nitroso-receptor. A possible role of the Ca^{2+}-binding protein, calmodulin, is obviously ruled out since the calmodulin-antagonistic compound trifluperazine[12] (0.1 mM) was without any influence on the observed Ca^{2+} inhibition (data not shown). Nevertheless, due to its steep concentration-response curve, minor changes in the level of free Ca^{2+} must have marked effects on guanylate cyclase activation by nitro-compounds. Hence, their guanylate cyclase stimulatory action appears to be sensitively modulated in vivo by intracellular variations of the free Ca^{2+} concentrations.

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Inhibition of brain enolases by acrylamide and its related compounds in vitro, and the structure-activity relationship

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Summary. Acrylamide and its related compounds inhibited brain enolases in vitro independently of their neurotoxicity. The inhibitory potency was a function of the binding constants of the compounds for phenylalanine. The binding constant for tryptophan was higher in neurotoxic compounds than in non-neurotoxic ones.
Key words. Rat brain; Enolase; acrylamide; acrylamide derivatives; neurotoxicity; structure-activity relationship.

It is well-known that acrylamide produces peripheral neuropathy in experimental animals and human beings[1,2]. Neurotoxicity has also been reported for some analogues, including N-hydroxymethylacrylamide[3-5], methacrylamide[4-6], N-isopropylacrylamide[4,5], and N-methylacrylamide[4,5] in mice and/or rats. Studies on the etiology of acrylamide neuropathy have suggested that inhibition of neuronal glycolytic enzymes such as enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by acrylamide might produce toxic distal axonopathies[7-12]. A recent study[13] in our laboratory, however, showed that both mouse brain total enolase and GAPDH were inhibited in vitro not only by neurotoxic acrylamide derivatives but also by non-neurotoxic ones. The present study was undertaken to examine further the inhibitory potency of the compounds for enolase isozymes in rat brain. The structure-activity relationship of the test compounds was also investigated in connection with their physico-chemical properties and the inhibitory potency on enolase.

Methods. Male Wistar rats (180–200 g) were used throughout the experiment. Three forms of brain enolase, a neuron-specific form ($\gamma\gamma$), a hybrid form ($\alpha\gamma$), and a non-neuronal form ($\alpha\alpha$), were separated by DEAE-cellulose column chromatography according to the method of Francis et al.[14]. Rats were killed by decapitation and brains were removed and homogenized (20% w/v) in cold 10 mM potassium phosphate buffer containing 1 mM magnesium sulfate, pH 7.4, using glass homogenizers fitted with Teflon pestles. Homogenates were centrifuged at 10,000 g for 20 min. The resulting supernatants were chromatographed using DEAE-cellulose (Whatman DE 32) column (25 × 2.0 cm). The column was washed successively with 3 portions of 10 mM phosphate buffer (1 mM $MgSO_4$, pH 7.4) containing 40, 130 and 240 mM KCl to elute each enolase. The