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Endothelin and a Ca^{2+} ionophore raise cyclic GMP levels in a neuronal cell line via formation of nitric oxide

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1 The vasoconstrictor peptide endothelin-1 caused a fast, transient rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in a neuronal cell line (mouse neuroblastoma x rat glioma hybrid cells 108CC15). The mechanism of activation of guanylate cyclase by endothelin-1 was investigated. The endothelin-1-induced rise depended on the release of internal Ca^{2+}.

2 The stimulation of cyclic GMP synthesis induced by endothelin-1 was suppressed after preincubating the cells in medium containing haemoglobin (IC_{50} 3 μM). Similarly, pretreatment of the cells with the L-arginine analogues, L-canavanine (IC_{50} 60 μM) or N^G-monomethyl-L-arginine (IC_{50} 2.5 μM), inhibited the cyclic GMP response to endothelin-1. Therefore, endothelin-1 activates guanylate cyclase most probably via formation of nitric oxide, which is released from L-arginine.

3 The Ca^{2+} ionophore ionomycin induced a transient rise in cyclic GMP levels, which was also suppressed by preincubation in the presence of either haemoglobin or the L-arginine analogues L-canavanine or N^G-monomethyl-L-arginine. Therefore, we conclude that ionomycin can activate guanylate cyclase by a mechanism involving nitric oxide formation, similar to that induced by endothelin-1.

4 The alkaloid veratridine, which activates Na^+ channels and also causes influx of Ca^{2+} induced a transient rise of cyclic GMP levels in the neuronal cell line. This stimulation was blocked by pretreating the cells with L-canavanine, N^G-monomethyl-L-arginine or haemoglobin.

5 Loading the cells with the Ca^{2+} chelator BAPTA suppresed the cyclic GMP response to application of endothelin-1, ionomycin, or veratridine. Thus, in the neuronal cell line a rise in cytosolic Ca^{2+} activity seems to be sufficient to stimulate the nitric oxide forming enzyme which synthesizes the activator of soluble guanylate cyclase.

Introduction

Several hormones and neurotransmitters raise guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in neural cell lines (Snider & Richelson, 1984; Reiser *et al.*, 1984; McKinney, 1987). Among the neurotransmitter receptors coupled to soluble guanylate cyclase are those for acetylcholine (muscarinic), histamine, 5-hydroxytryptamine and various peptides like neurotensin and bradykinin (Waldman & Murad, 1987). The functional consequences of increases in cyclic GMP levels in neural tissue are largely unknown. Apart from the well established function of cyclic GMP in the visual transduction process, there is little evidence of a role of cyclic GMP in regulation of ion channels (Stryer, 1986).

Furthermore, the mechanism of activation of guanylate cyclase by the diverse neurotransmitter receptors is still a matter of controversy. It has been suggested that arachidonic acid metabolites formed by lipoxygenase activity are involved in activation of guanylate cyclase (Snider *et al.*, 1984; McKinney & Richelson, 1986; Friedl, 1986; McKinney, 1987). However, recently it has been established that endothelium-derived relaxing factor (EDRF, Furchgott & Vanhoutte, 1989), which has been identified as nitric oxide (Palmer *et al.*, 1987), is active in a variety of systems in which soluble guanylate cyclase is stimulated (Moncada *et al.*, 1989). Thus, nitric oxide or a related nitroso compound seems to be a second messenger of widespread significance (Knowles *et al.*, 1989).

Here, evidence is presented that the vasoconstrictor peptide endothelin-1 (Yanagisawa *et al.*, 1988) induces a rise in cyclic GMP levels in a neuronal cell line mediated by nitric oxide or a related nitroso compound. Moreover, it is shown that the Ca^{2+} ionophore ionomycin causes a transient rise in cyclic GMP levels, which is also due to formation of nitric oxide. Veratridine, which indirectly induces influx of Ca^{2+} into cells is demonstrated to stimulate cyclic GMP synthesis by the same mechanism.

Methods

Measurement of cyclic GMP levels

Mouse neuroblastoma x rat glioma hybrid cells, clone 108CC15 of passage numbers between 14 and 32, were cultured as described (Hamprecht *et al.*, 1985). Cells were seeded at a density of 3 to 4×10^5 cells in plastic Petri dishes (diameter 50 mm) and grown for 2 or 3 days. To start the experiment, the growth medium was removed and the cells were washed twice with 2 ml incubation medium containing (mM): NaCl 145, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, Na_2HPO_4 2.0, glucose 20 and HEPES 20, adjusted to pH 7.4 with Tris, osmolarity 320–350 mOsmol l^{-1}. Cells were preincubated at 37°C in 2 ml incubation medium for a period of 25 to 35 min allowing the cells to equilibrate. This preincubation has been found to enhance the sensitivity of the hybrid cells to peptides stimulating cyclic GMP levels (Friedl, 1986). Reactions were started by adding 20 μl of a concentrated stock solution of the compounds to be tested, dissolved in incubation medium or in H_2O. Veratridine was added as a 0.4 mM solution to give final concentrations between 0.1 and 0.2 mM. Usually after 20 s, reactions were stopped by adding 1 ml ethanol. Cellular content of cyclic GMP was determined with incubations carried out in duplicate by radioimmunoassay as described by Reiser *et al.* (1984), and referred to cellular protein, determined by the Lowry method using bovine serum albumin as standard. Oxyhaemoglobin was prepared by adding a molar excess of dithionite to a solution of haemoglobin, bubbling through with O_2 and desalting by Sephadex G-25 (Pharmacia, Uppsala, Sweden) chromatography. The stability of oxyhaemoglobin was monitored spectrophotometrically after the experiment (absorption maxima at 576, 540 and 410 nm).

Materials

Bradykinin triacetate, the bradykinin BK_2 antagonist [$Thi^{5,8}$, D-Phe^7]-bradykinin (H-Arg-Pro-Pro-Gly-Thi-Ser-D-Phe-Thi-

Arg-OH), haemoglobin (bovine), veratridine and canavanine were from Sigma (München, F.R.G.); endothelin (endothelin-1) from Bachem Biochemica (Heidelberg, F.R.G.); ionomycin and N^G-monomethyl-L-arginine were from Calbiochem (Frankfurt, F.R.G.); BAPTA (*bis*(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid)/acetoxymethylester was from Molecular Probes (Eugene, OR, U.S.A.). D888 (desmethoxyverapamil) was kindly provided by Dr Traut from Knoll A.G. (Ludwigshafen, F.R.G.). All other chemicals, of analytical grade, were purchased from E. Merck (Darmstadt, F.R.G.) or Sigma (Deisenhofen, F.R.G.).

Results

Endothelin-1 induced a rise in cyclic GMP levels in the neuroblastoma x glioma hybrid cells. The cyclic GMP levels rose to a maximum within 30 s after addition of the peptide and declined sharply thereafter (Figure 1a). The baseline value was reached again 60 s after the beginning of the challenge. The concentration-response curve (Figure 1b) shows that endothelin-1 raises cyclic GMP levels at concentrations above 10 nM, with a half-maximal value at 55 nM. The maximum was reached at concentrations above 200 nM.

The capacity of the cells to respond to endothelin-1 was lost at extracellular Ca^{2+} concentrations below 100 μM (Figure 1c) when cells were preincubated in media with various Ca^{2+} concentrations for 30 min. However, after only 5 min preincubation in medium with 10 and 50 μM Ca^{2+} the stimulation by 100 nM endothelin-1 was still 51 and 76% of that under control conditions, respectively (data not shown).

In a series of experiments the neuronal cells were preloaded with the Ca^{2+} chelator BAPTA. The cells were preincubated for 30 min in the presence of varying concentrations of the membrane permeant analogue BAPTA acetoxymethylester, which is cleaved intracellularly. Thus, free BAPTA accumulates in the cells. Subsequent challenges with endothelin-1 resulted in reduced rises in cyclic GMP levels. Half-maximal inhibition was seen at 2.5 μM BAPTA in the incubation medium (not illustrated).

When the cells were pretreated for 20 to 30 min in incubation medium containing the Ca^{2+} ionophore ionomycin at concentrations below 1 μM (Figure 1d(iv)) the cells could no longer be stimulated by endothelin-1. The comparable inhibition of bradykinin-stimulated cyclic GMP increase by pretreatment with ionomycin is also displayed in Figure 1d(ii). Addition of the organic Ca^{2+} channel antagonist D888 (desmethoxyverapamil) did not affect the cyclic GMP response to endothelin-1 at concentrations up to 50 μM (Figure 1d(iii)). The neuroblastoma x glioma hybrid cells display functional receptors for the neuropeptide bradykinin (Reiser & Hamprecht, 1985; Reiser *et al.*, 1990). [Thi^{5,8}, D-Phe^7]-bradykinin, a BK_2 receptor antagonist, at 10 μM suppressed the effect of bradykinin almost completely, but did not affect the stimulation by endothelin-1 (Figure 1d(iv)).

Methylene blue, an agent which inhibits soluble guanylate cyclase (Waldman & Murad, 1987), blocked the rise in cyclic GMP levels induced by 100 nM endothelin-1 (Figure 2a), half-maximally at 4 ± 1 μM (mean ± s.d., $n = 3$). Oxyhaemoglobin blocked the rise of cyclic GMP levels induced by endothelin-1 (Figure 2b) with half-maximal effect at 3 ± 1 μM. Preincubating the hybrid cells with the arginine analogue L-canavanine suppressed the ability of endothelin-1 to raise the cyclic GMP levels (Figure 2c) with an IC_{50} value of 60 ± 36 μM ($n = 3$). N^G-monomethyl-L-arginine blocked the rise in cyclic GMP levels caused by endothelin-1 (Figure 2c) with an IC_{50} of 2.5 ± 1.4 μM ($n = 4$).

The Ca^{2+} ionophore ionomycin at concentrations above 100 nM caused a transient rise in cyclic GMP levels in the hybrid cells. The maximum rise was obtained 20 s after addition of ionomycin (Figure 3, a–c). Figure 3 also demonstrates that the stimulation by ionomycin could be suppressed by L-

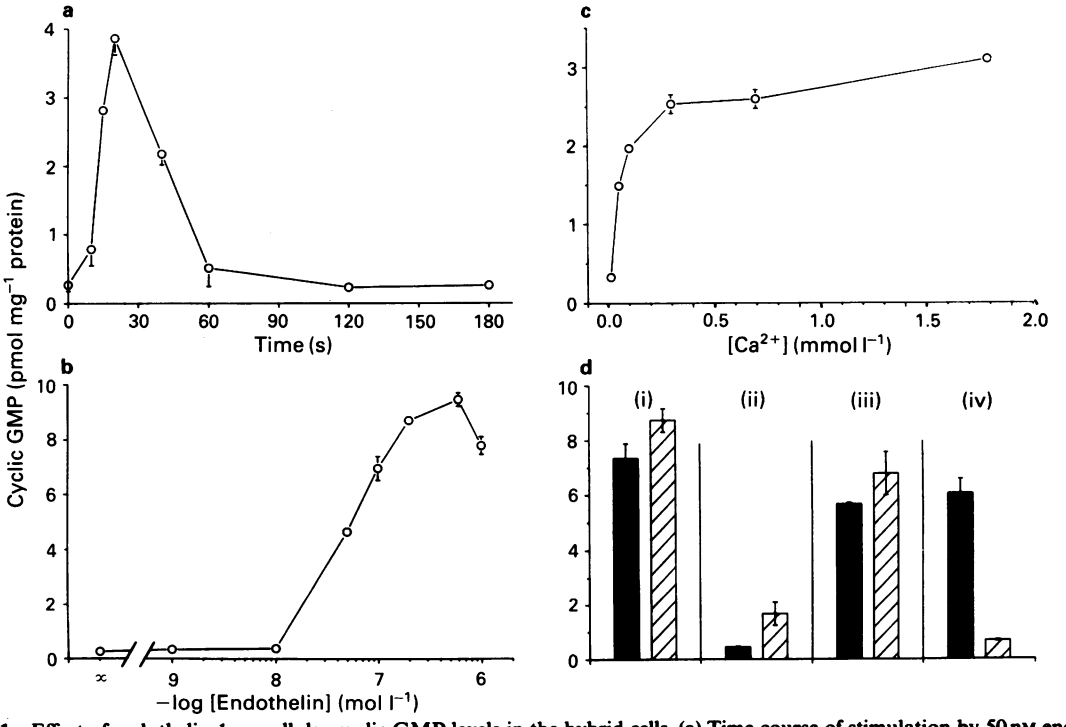

Figure 1 Effect of endothelin-1 on cellular cyclic GMP levels in the hybrid cells. (a) Time course of stimulation by 50 nM endothelin-1. (b) Concentration-effect curve. Cells were challenged with various concentrations of endothelin-1 for 20 s. In (a) and (b) after a 30 min preincubation in incubation medium, endothelin-1 was added and cellular cyclic GMP levels were determined as described in Methods. (c) Influence of extracellular Ca^{2+} concentration on stimulation of cyclic GMP levels by endothelin-1. Cells were preincubated for 30 min in medium containing various Ca^{2+} concentrations. Then the cells were challenged with 100 nM endothelin-1 for 20 s. (d) Stimulation of cyclic GMP levels by 100 nM endothelin-1 (solid columns) or 100 nM bradykinin (hatched columns). Before exposure to the peptides the cells were preincubated for 30 min in incubation medium (i) without addition for control, or supplemented with (ii) 100 nM ionomycin, (iii) 50 μM D888, or (iv) 10 μM bradykinin BK_2 antagonist [Thi^{5,8}, D-Phe^7]-bradykinin (H-Arg-Pro-Pro-Gly-Thi-Ser-D-Phe-Thi-Arg-OH). (a) and (b) are taken from the same experiment. Here and in Figures 2 and 3 typical results are shown, which are representative of at least two experiments with comparable results. Basal content of cyclic GMP was 0.3 ± 0.2 pmol mg^{-1} protein.

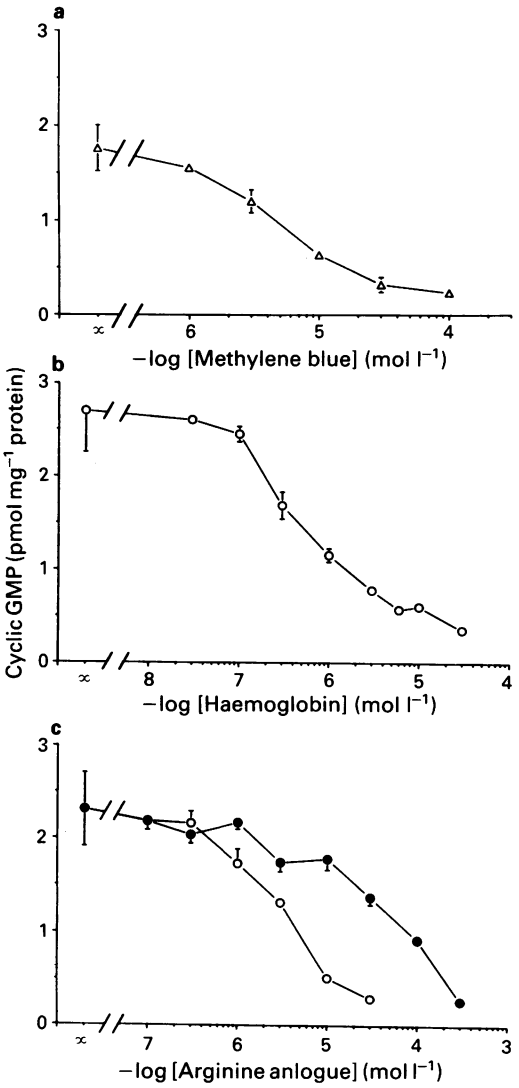

Figure 2 Inhibition by methylene blue (a), oxyhaemoglobin (b) and the arginine analogues (c) N^G-monomethyl-L-arginine (○) and canavanine (●), of cyclic GMP responses of hybrid cells induced by 100 nM endothelin-1. Various concentrations of methylene blue were added during the preincubation. Haemoglobin and L-arginine analogues were given for the last 2.5 min of the preincubation period, and subsequently the cells were challenged with endothelin-1 for 20 s.

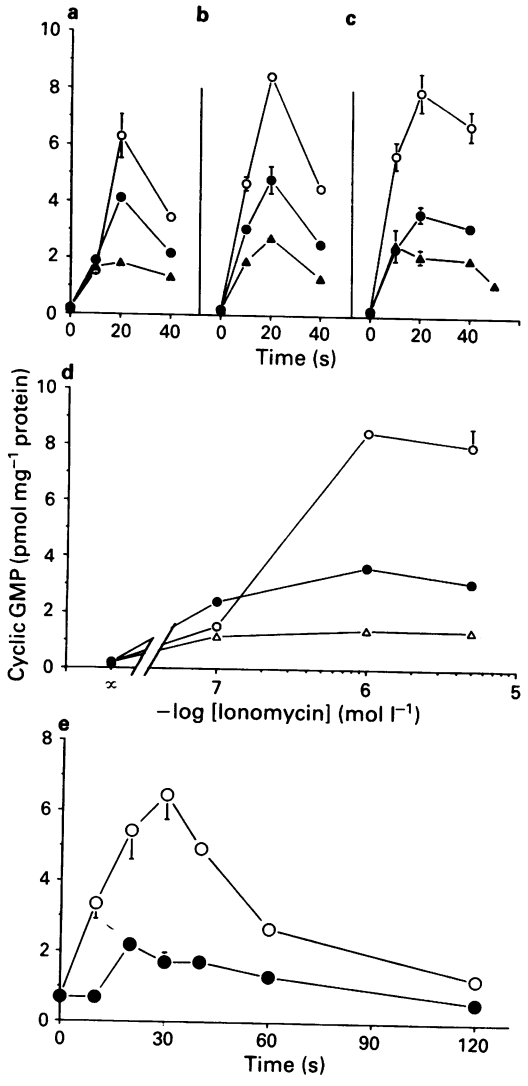

Figure 3 Influence of ionomycin (a–d) and veratridine (e) on cyclic GMP levels in the hybrid cells. Cells were challenged for the periods of time indicated on the abscissa scale in incubation medium containing ionomycin at concentrations of 0.1 μM (a), 1 μM (b) and 5 μM (c) in the absence (○) or presence of 100 μM canavanine (●) or 10 μM N^G-monomethyl-L-arginine (△). (d) Rise of cyclic GMP levels induced by various concentrations of ionomycin (challenge incubation period 20 s) without addition (○) or with 1 μM (●) or 10 μM haemoglobin (△). (e) Time course of stimulation of cyclic GMP level by 0.2 mM veratridine. Cells were tested after 30 min preincubation in medium without addition (○) or with addition of 6 μM of N^G-monomethyl-L-arginine (●) for the last 2.5 min of the preincubation period.

canavanine or by N^G-monomethyl-L-arginine. Half-maximal inhibitory activity was seen at 6 ± 5 μM ($n = 3$) for N^G-monomethyl-L-arginine and at 60 ± 50 μM ($n = 3$) for L-canavanine (data not shown). The rise in cyclic GMP levels caused by ionomycin was also inhibited by incubating the cells in the presence of oxyhaemoglobin (Figure 3d) with half-maximal inhibition at 0.6 μM ($n = 2$).

Veratridine (0.2 mM) induced a rise in cyclic GMP levels in the neuronal cell line with the maximum reached after 30 s (Figure 3e). The decline towards the basal levels was slower than following stimulation with endothelin-1 such that there was still a significant elevation of cyclic GMP levels at 60 s. As can also be seen in Figure 3e, N^G-monomethyl-L-arginine (6 μM) blocked the veratridine-induced rise in cyclic GMP levels. Half-maximal inhibition was obtained at 3 ± 2 μM N^G-monomethyl-L-arginine ($n = 3$) and 50 ± 45 μM canavanine ($n = 3$). The effect of 0.2 mM veratridine was also suppressed by haemoglobin (IC_{50} 0.5 μM) or by pretreating the cells with BAPTA (IC_{50} 4 μM; data not shown).

Discussion

In a neuronal cell line endothelin-1 activates guanylate cyclase with a time course similar to the effects of bradykinin (Reiser *et al.*, 1984) on BK_2 receptors and of 5-hydroxytryptamine on 5-HT_3 receptors (Reiser & Hamprecht, 1989; Reiser, 1990). Endothelin-1 does not act on the bradykinin receptors since a BK_2 receptor antagonist suppressed the stimulation by bradykinin but not by endothelin. Blockade by haemoglobin or by methylene blue indicates that the mediator between activated endothelin-1 receptor and soluble guanylate cyclase resembles EDRF, which has been identified as nitric oxide (Palmer *et al.*, 1987). Haemoglobin seems to scavenge an activating substance that is released into the extracellular space after challenge with endothelin-1. Thus a diffusible factor released from the cells could act in a paracrine manner, as suggested for cerebellar granule cells exposed to N-methyl-D-asparate (Garthwaite *et al.*, 1988).

Nitric oxide or a nitroso compound has been reported to be formed enzymatically from terminal guanidino nitrogen of L-arginine (Schmidt *et al.*, 1988; Palmer *et al.*, 1988; Moncada *et al.*, 1989). This synthesis of nitric oxide can be suppressed by structural analogues of L-arginine, such as N^G-monomethyl-L-

arginine and L-canavanine (Schmidt *et al.*, 1988). Since both analogues effectively block the rise of cyclic GMP levels caused by endothelin-1, formation of nitric oxide from L-arginine appears to be involved in the action of endothelin-1 in the hybrid cells.

Endothelin-1 raises cytosolic Ca^{2+} activity transiently for about 1 min in the neuronal hybrid cells loaded with the Ca^{2+}-sensitive dye fura-2 (Reiser & Donié, 1990) as in glial cells (Ashley *et al.*, 1989). The rise in cytosolic Ca^{2+} activity is mediated by release from internal stores through inositol 1,4,5-trisphosphate as we have demonstrated by the following findings: (i) Endothelin-1 caused a rise of cytosolic Ca^{2+} activity even in the absence of external Ca^{2+} and (ii) depletion of internal Ca^{2+} stores by ionomycin abolished the Ca^{2+} effect of endothelin-1 (Reiser & Donié, 1990).

The stimulation of guanylate cyclase by endothelin-1 depends on a rise of cytosolic Ca^{2+} activity, since buffering of cytosolic Ca^{2+} by BAPTA and depletion of internal stores by pretreatment with the Ca^{2+} ionophore, ionomycin, suppressed the cyclic GMP response (Figure 1d(ii)). At the low concentrations used the Ca^{2+} ionophore primarily depletes internal Ca^{2+} stores and prevents their refilling (Pollock *et al.*, 1987; Reiser *et al.*, 1989; 1990). The inhibition seen at low extracellular Ca^{2+} concentrations (Figure 1c) seems to be due to a depletion of internal Ca^{2+} stores, since the size of inhibition decreases with shortening the length of the preincubation period in Ca^{2+}-depleted medium.

A rise in cytosolic Ca^{2+} activity appears to be the crucial step for activation of guanylate cyclase in the neuronal hybrid cells, since buffering intracellular Ca^{2+} by the chelator BAPTA (Grynkiewicz *et al.*, 1985) suppressed the stimulation either by endothelin-1, ionomycin or veratridine. Cyclic GMP synthesis is activated similarly by bradykinin via BK_2 receptors which primarily release Ca^{2+} from internal stores (Reiser *et al.*, 1990), or by 5-hydroxytryptamine via 5-HT_3 receptors (Reiser & Hamprecht, 1989), which induce influx of extracellular Ca^{2+} (Reiser *et al.*, 1989).

Here we show that the Ca^{2+} ionophore, ionomycin, causes a transient rise of cyclic GMP levels. The time course is comparable to that of the rise of cytosolic Ca^{2+} activity induced by ionomycin in the hybrid cells (Reiser *et al.*, 1989). The mechanism of activation of guanylate cyclase by ionomycin seems to employ nitric oxide, like the activation by the hormones bradykinin, 5-hydroxytryptamine (Reiser, 1990) or endothelin-1 (shown here). If the Ca^{2+} ionophore has no direct effect on any enzymatic activity in the neuronal cells, we can conclude that a rise in cytosolic Ca^{2+} activity seems to be sufficient to activate the nitric oxide forming enzyme. The Ca^{2+}-requirement of the cytosolic enzyme which forms nitric oxide (Knowles *et al.*, 1989) is due to the influence of calmodulin (Bredt & Snyder, 1990). Further support for our interpretation comes from the ability of veratridine to increase cyclic GMP levels in the hybrid cells (Figure 3). The alkaloid veratradine, which activates voltage-dependent Na^+ channels (Ulbricht, 1969) and induces oscillatory depolarizations of the membrane of the hybrid cells (Reiser & Hamprecht, 1983), indirectly causes a rise in Ca^{2+} influx in neuroblastoma cells (Freedman *et al.*, 1984).

Thus, all the agents studied here, which raise cyclic GMP levels, namely endothelin-1, ionomycin and veratridine, cause a rise in cytosolic Ca^{2+} activity. The increase in cytosolic Ca^{2+} activity seems to be a prerequisite for the cascade of nitric oxide formation and subsequent activation of guanylate cyclase. The cascade can be interrupted either by chelating cytosolic Ca^{2+} or by blocking nitric oxide formation.

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