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Endogenous inotropic factor-induced endothelium-dependent relaxation of vascular smooth muscle

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1 Possible contractile or relaxation effects of an endogenous inotropic factor (EIF) isolated and purified from porcine heart left ventricle were examined in rat isolated aortic ring preparations.

2 EIF induced a dose-dependent relaxation of the rat isolated aortic ring preparation pre-contracted with 0.4 μM phenylephrine (PE); 200 μl (in 5 ml bath) of EIF caused relaxation of aortic rings by as much as 67.4±4.5%. In another set of experiments, in the presence of 100 μl EIF, the PE concentration-response contractile curve shifted to the right, the maximal contractile force was reduced by as much as 32.8% and the EC_{50} of PE increased from 0.2 to 0.3 μM.

3 The relaxation effect of EIF was demonstrated to be endothelium-dependent. Additional experiments demonstrated that EIF-induced relaxation in an isolated aortic ring could be inhibited by 2 μM N^G-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, suggesting the involvement of nitric oxide in EIF-induced relaxation of the muscle.

4 Atropine (0.2 μM) or indomethacin (10 μM) had no significant effect on EIF-induced relaxation.

5 These data suggest that EIF, a novel endogenous inotrope from porcine myocardium, also acts as an endothelium-dependent vasodilator substance mediating relaxation in the rat isolated aorta mainly by release of nitric oxide. The possibility of EIF acting through muscarinic receptor and the involvement of prostacyclin were excluded.

Keywords: Endogenous factor; smooth muscle; relaxation; nitric oxide; phenylephrine

Introduction

We have previously reported isolation of an endogenous factor from porcine heart left ventricle (Khatter *et al.*, 1986). This factor was characterized pharmacologically as an endogenous inotropic factor (EIF). We have also demonstrated that EIF, in spite of its positive inotropic effect on cardiac preparations, lacks digitalis-like cardiac toxicity (Navaratnam *et al.*, 1990) and only weakly cross-reacted with digitoxin anti-serum (Khatter *et al.*, 1991). More recently, after several steps of purification of the crude fraction (Agbanyo & Khatter, 1990; Navaratnam *et al.*, 1990; Khatter *et al.*, 1991), we demonstrated that one of the possible mechanisms of the EIF inotropic effect may be an increased transmembrane calcium influx (Chen *et al.*, 1993).

The effect of EIF on vascular smooth muscle has not been previously studied as to whether it alters vascular smooth muscle tone. To address this question, we examined the effect of EIF on rat isolated aortic smooth muscle and studied the possible mechanism of action.

Methods

Extraction and purification of EIF

EIF used in this study was extracted according to the method published previously (Khatter *et al.*, 1986). Briefly, fresh porcine left ventricle, cleared of blood and fat, was minced and homogenized. The homogenate was extracted in acetone acidified with HCl. The supernatant fraction was delipidated and further purified by ion-exchange and silica columns (Agbanyo & Khatter, 1990; Khatter *et al.*, 1991). Additional purification was carried out by reverse phase high performance liquid chromatography (h.p.l.c.). The purified fraction was dissolved in distilled water and neutralized to pH 7.4. The final solution containing EIF equivalent to 100 μl per 3 g of extracted tissue was stored at −20°C until use.

Isolated aortic smooth muscle preparation

Sprague-Dawley rats of either sex, weighing 250-300 g were used in this study. The animals were killed by decapitation and the thoracic aortae were removed surgically. After connective tissues were cleaned off, the aortae were placed in a beaker containing modified Krebs-Henseleit buffer (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.4, Na_2CO_3 25, glucose 11 and HEPES 5) which was continuously gassed with carbogen. The aortae were cut into 3-4 mm transverse rings. In preparing the aortae, great care was taken to protect the endothelium from being damaged. In one set of the experiments, the endothelial cells were denuded intentionally to ascertain the role of the endothelium. To remove the endothelial cells, the luminal surface of the aortic rings was scraped mechanically with a piece of stainless steel wire. During the preparation of both endothelium-intact and denuded tissue, the aortic rings were frequently washed with oxygenated buffer.

The tissue preparations were suspended in a 5 ml water jacketed bath by two stainless steel hooks. The bath containing buffer solution was continuously bubbled with carbogen at 36.5±0.5°C. One of the hooks was mounted at the bottom of the bath while the other was coupled to a Grass (Quincy, MA) FT03 force transducer to measure the isometric tension. Isometric force was recorded on a Beckman (Schiller Park, II) model 511A Dynograph recorder. One gram basal tension was applied in all experiments.

After 1 h equilibration, the tissues were pre-exposed to phenylephrine (PE, 4 μM) for 5 min. The drug was then washed off three times at 3 min intervals, followed by 4-5 washes, each

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10 min apart. The basal tension was always monitored and adjusted to 1 g. The tissues were allowed to equilibrate further for 5–10 min before experiments were started.

Experimental protocols

Effect of EIF in PE pre-contracted aortic rings The tissues were contracted by the addition of 0.4 μM PE. Five minutes later (after stabilization of contractile force), cumulative doses of EIF (20–200 μl) were added. The tissues were allowed to equilibrate at each of the doses for 4–5 min to obtain stable contractility. In another set of experiments, 100 μl of EIF was added 5 min before the addition of PE. Concentration-response curves were then constructed with different concentrations of PE (2.0 nM to 2.6 μM). Each additional aliquot of PE was added at a 5 min interval. In all control groups, the same amount of distilled water was used instead of EIF.

The role of endothelium in the effect of EIF Both endothelium-denuded and endothelium-intact tissues were pre-contracted with PE as described above. Doses of 50 and 150 μl of EIF and 0.6 μM nitroglycerin (GTN) were then added cumulatively (5 min apart). PE concentration-response curves were also constructed with endothelium-denuded tissues by following the procedure described above. PE concentrations ranged from 2.0 nM to 2.2 μM.

The effects of N^G-nitro-L-arginine melthyl ester (L-NAME) and L-arginine (L-Arg) on acetylcholine (ACh) and EIF-induced relaxation The tissues were pre-contracted with PE as described before. After 5 min of equilibration, 0.2 μM ACh or 50 and 200 μl EIF were added to three different groups of isolated aortic muscle preparations and the changes in contractile force were recorded. The drugs were then washed out by three short washes (3 min apart) followed by 4–5 10 min washes. After stabilization of the basal tension, 2.0 μM L-NAME was added to all three groups of isolated tissue preparations. Ten minutes later, the tissues were contracted with PE (0.4 μM) and the relaxation effects of the same concentrations of ACh or EIF were tested. The tissues were washed for the second time. After a stabilization period, L-NAME (2.0 μM), and 3 min later 0.2 mM L-Arg were added to all three groups of tissue preparations. Seven minutes later, the tissues were contracted with PE and the effect of ACh or two doses of EIF was once again tested.

The effect of atropine on ACh and EIF-induced relaxation The tissues were pre-contracted with PE (0.4 μM) as before. After stabilization of contractility, atropine (0.2 μM) was added to the contracted tissues. Two minutes later, ACh (0.2 μM) or EIF (50 and 200 μl) were added and the changes in contractility recorded.

Effect of indomethacin The tissues were treated with 10 μM indomethacin 5 min before contraction with PE. EIF (200 μl) was then added and the changes in contractility recorded.

Drugs

Phenylephrine, N^G-nitro-L-arginine methyl ester, L-arginine and acetylcholine chloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Atropine sulphate was obtained from Abbott Laboratories, Ltd. (Montreal, Quebec, Canada). Nitroglycerin was obtained from Parke-Davis Canada Inc. (Scarborough, Ont., Canada). All of the above drugs were dissolved in distilled water as stock solutions and were diluted to desired concentrations in Krebs-Henseleit buffer right before each experiment. Indomethacin, obtained from Sigma, was dissolved in 20 mM Na_2CO_3 solution.

Data analysis

The relaxation effects were expressed as percentage relaxation from the PE-induced contractile state of the aortic smooth muscle. The PE concentration-response curves were expressed as contractile force in g. The data are presented as mean ± s.e. Student's *t* test and analysis of variance were used and $P<0.05$ was selected as the criterion for a statistically significant difference.

Results

Cumulative doses of EIF (from 20 to 200 μl) induced a dose-dependent relaxation of rat aortic smooth muscle rings pre-contracted by 0.4 μM PE (EIF versus controls with 40, 1 degree of freedom (d.f.), $P<0.005$; EIF different dose with 20, 1 d.f., $P<0.005$). Increases in contractile force from basal levels induced by 0.4 μM PE were not significantly different in the different preparations as long as the width of the aortic rings was tightly controlled. As little as 20 μl EIF induced 10.3 ± 1.9% relaxation of these rings from the pre-contracted state. When the dose of EIF accumulated to 200 μl, as much as 67.4 ± 4.5% relaxation of the tissues was observed (Figure 1) which was significantly higher than that with 20 μl of EIF. In the absence of PE, EIF had no influence on the basal tone of the smooth muscle preparations at the doses used above. To investigate if the EIF-induced relaxation of rat isolated aortic smooth muscle was endothelium-dependent, PE concentration-response curves were constructed using endothelium-intact and -denuded tissues. The PE concentration-response curves in the presence of vehicle (distilled water) or 100 μl EIF were compared. The lower two curves in Figure 2 represent PE concentration-response curves of aortic smooth muscle with intact endothelium. In the presence of 100 μl EIF, the responses to PE were attenuated resulting in a shift to the right of the response curve (EIF versus control with 60, 19 d.f., $P<0.005$). The statistical analysis indicated that the significant differences between the two curves existed at PE concentrations of 0.2 μM and up. The maximal contractile force decreased by 32.8%. The EC_{50} of PE was also increased from 0.2 to 0.3 μM in the presence of EIF. The upper two curves of the PE concentration-response in Figure 2 were constructed using endothelium-denuded tissues. In endothelium-denuded preparations, the maximal contractility of the tissue was 24.9% higher from that of endothelium-intact preparations and the EC_{50} of PE decreased from 0.2 μM to 6.7 nM. EIF had no influence whatsoever on endothelium-denuded preparations.

Figure 3 shows that the relaxation effect of 50 and 200 μl EIF on PE-contracted aortic smooth muscle preparations was

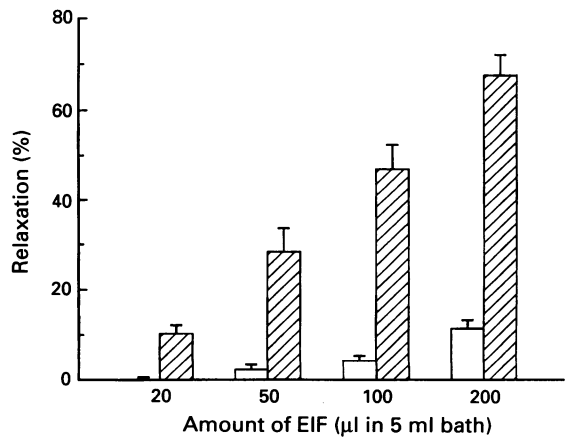

Figure 1 Effect of EIF on PE pre-contracted aortic smooth muscles. Cumulative doses of EIF were added to 0.4 μM PE pre-contracted tissues (hatched columns). In control experiment, EIF was replaced by distilled water (open columns). The data are mean ± s.e. of 6 experiments. (EIF versus control, $P<0.005$; EIF dose-dependency, $P<0.005$).

totally abolished when the endothelial cells were removed manually. These endoethelium-denuded tissues, however, were still sensitive to addition of GTN (0.6 μM), which induced about 95% relaxation of the tissue preparations from the pre-contracted state. Furthermore, in endothelium-intact aortic preparations, EIF-induced and GTN-induced relaxations were additive.

ACh (0.2 μM) induced 62.0±12.3% relaxation of PE-contracted aortic smooth muscle preparations. This relaxation effect of ACh was significantly inhibited by addition of 2.0 μM L-NAME to the tissue ($n=4$, $P<0.01$). On addition of excess of L-Arg (0.2 mM), the relaxant effect of ACh was completely restored (Figure 4). Similar observations were made on the relaxation induced by 50 and 200 μl EIF (Figure 4). L-NAME (2.0 μM) also significantly inhibited the relaxation induced by both doses of EIF (for 50 μl EIF, $n=6$, $P<0.05$; for 200 μl EIF, $n=5$, $P<0.01$), which were restored to near control levels when excess of L-Arg (0.2 mM) was added.

The addition of atropine (0.2 μM) had no influence on the contractility of aortic rings induced by PE. However, the relaxation effect of ACh was totally blocked by the addition of 0.2 μM atropine (Figure 5). Atropine had no effect on EIF-induced relaxation suggesting that muscarinic receptors were not involved in EIF-induced relaxation of rat aortic smooth muscle. In another study it was found that treatment of aortic tissues with 10 μM indomethacin had no influence on EIF-induced relaxation of the tissue, suggesting that prostacyclins were not involved in EIF-induced relaxation of rat aortic smooth muscle.

Discussion

The present study demonstrated, for the first time, that a novel endogenous inotropic factor (EIF) isolated from pig myocardium also acts as an endothelium-dependent vasodilator

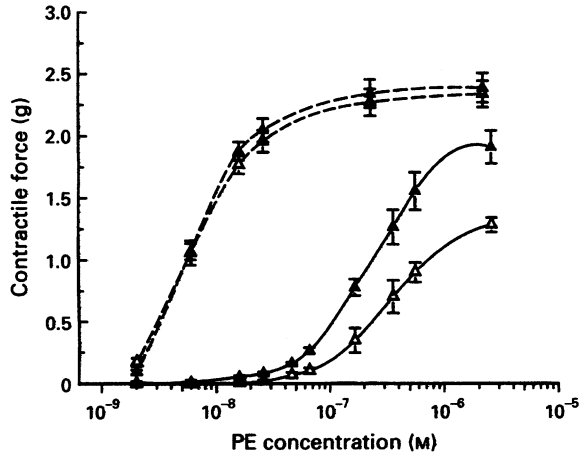

Figure 2 Effect of EIF on PE-induced contractions. Lower two curves: PE concentration-response curves of endothelium-intact tissues in the presence of 100 μl EIF (△) or water (▲). The data are mean±s.e. of 4 experiments. (EIF versus control, $P<0.005$. Statistical analysis: the difference exists at PE concentration of 0.2 μM and higher). Upper two curves: PE concentration-response curves of endothelium-denuded tissues in the presence of 100 μl EIF (△) or water (▲). The data are mean±s.e. of 4–5 experiments. (EIF versus control, NS).

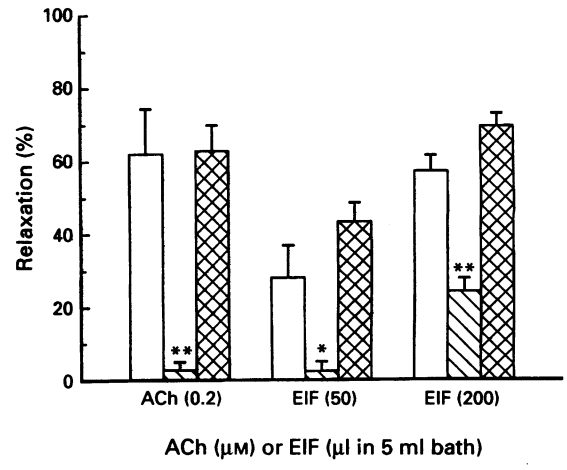

Figure 4 Antagonism of ACh and EIF-induced relaxation by L-NAME. The relaxation effects of ACh (0.2 μM) and EIF (50 and 200 μl) on PE pre-contracted tissues (open columns) were compared to those in the presence of L-NAME (2 μM, hatched columns) or L-NAME and excess of L-Arg (0.2 mM, cross-hatched columns). The data are mean±s.e. of 4–6 experiments. (* $P<0.05$; ** $P<0.01$).

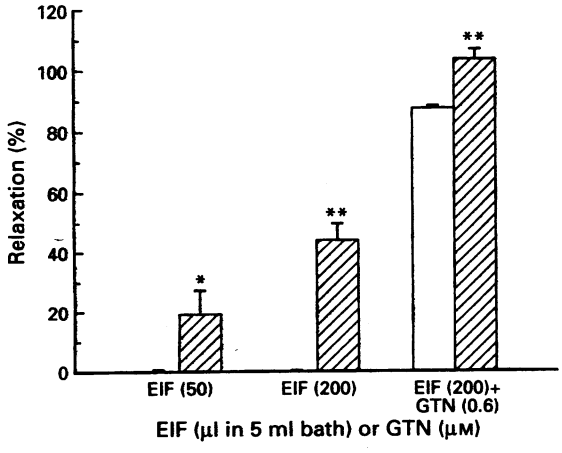

Figure 3 Role of endothelial cells in EIF-induced relaxation. Endothelium-denuded (open columns) and intact (hatched columns) tissues were used in this study. Doses of EIF (50 and 150 μl) and one concentration of GTN (0.6 μM) were added cumulatively to PE pre-contracted tissues. Each column represents mean±s.e. of 3 experiments. (* $P<0.05$, ** $P<0.01$).

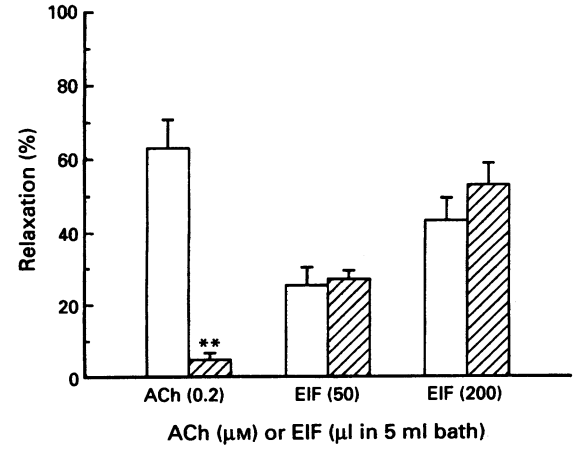

Figure 5 Effect of atropine on ACh and EIF-induced relaxation. The relaxation effect of ACh (0.2 μM) and EIF (50 and 200 μl) on PE pre-contracted tissues was compared in the absence (open columns) and presence (hatched columns) of 0.2 μM atropine. The data are mean±s.e. of 4 experiments. (** $P<0.01$).

substance, mediating relaxation in the rat isolated aorta mainly by release of nitric oxide. The dose-range of EIF which we demonstrated to have a relaxant effect was the same as that used in the study to demonstrate its inotropic effect (Navaratnam *et al.*, 1990). The results confirmed that the relaxation effect on smooth muscle is another important property of EIF.

The physiological importance of the endothelium has been demonstrated by Furchgott & Zawadzki (1980). The studies revealed the obligatory role of endothelial cells in ACh-induced vascular smooth muscle relaxation. The endothelium-derived relaxing factor (EDRF) (for review see Angus & Cocks, 1989; Furchgott, 1993) was suggested to be nitric oxide (Furchgott, 1988, for review see Moncada *et al.*, 1991). In order to determine whether the endothelium-dependent relaxation effect of EIF was nitric oxide-mediated, L-NAME, an analogue of L-Arg which inhibits nitric oxide synthase was used in our study. L-NAME has successfully been used to inhibit ACh induced relaxation *in vitro* and the vasodilatation response *in vivo* (Wang *et al.*, 1993). Our data are in agreement with these results and also demonstrate the inhibition of EIF-induced relaxation of rat aortic smooth muscle by L-NAME. Furthermore, EIF-induced relaxation could be restored with excess L-Arg. These observations clearly suggest an involvement of nitric oxide in EIF-induced and endothelium-dependent relaxation in rat isolated aortic smooth muscle. That ACh produces vascular smooth muscle relaxation via nitric oxide synthesis is supported by many studies (Palmer *et al.*, 1988; Crawley *et al.*, 1990; Rees *et al.*, 1990). ACh binding to the muscarinic receptors on the endothelial cell membranes leads to an increased cytosolic calcium concentration, which consequently activates the nitric oxide synthase and results in release of nitric oxide and hence relaxation of smooth muscle cells (for review see Knowles & Moncada, 1992; Moncada & Higgs, 1993). An increased intracellular calcium concentration seems to be the key step in terms of signal transduction (Angus & Cocks, 1989). Our earlier studies have suggested that the inotropic effect of EIF in cardiac muscle may be secondary to an influx of calcium and thus an increased intracellular concentration (Chen *et al.*, 1993). The apparently opposite effects of EIF on cardiac muscle and aortic smooth muscle may be closely linked by the intracellular calcium. That EIF did not change the basal tone of endothelium-denuded smooth muscle preparations suggested that the site of action of EIF could not be directly on the smooth muscle cells in our model. One can assume that there are specific receptors for EIF which, like muscarinic receptors, can signal an increase in intracellular calcium. Such receptors may be located or much more concentrated on the endothelial cell membranes. EIF does not act through muscarinic receptors as atropine, a muscarinic receptor blocker which fully blocked the effect of ACh, had no influence on EIF-induced relaxation. The question whether EIF has its own receptors through which a signal transduction takes place to increase intracellular calcium still remains to be investigated.

An interesting observation in our experiments was that the endothelium-denuded tissue preparations were more sensitive to PE. When the endothelial cells were destroyed mechanically, the EC_{50} of PE decreased by about 33 fold (from 0.2 μM to 6.7 nM). The maximal contractile force developed by PE increased by 24.9% in endothelium-denuded preparations. This phenomenon may suggest that relaxing factors which are derived from endothelial cells physiologically oppose the increase in tension of the tissues. The mechanisms by which an increased tension leads to an increased release of relaxant factors from the endothelium of isolated aortic smooth muscle were not studied.

The involvement of nitric oxide in EIF-induced relaxation of rat aortic smooth muscle preparations has been discussed earlier in this paper. However, the degree of antagonism of EIF (200 μl)-induced relaxation by L-NAME was much less than that to the nearly identical relaxation induced by ACh (57.6 versus 95.6% inhibition from their controls, $n=3$-4, $P<0.005$, Figure 4). It is conceivable that some mechanism besides nitric oxide release may be involved in EIF-induced endothelium-dependent relaxation, especially with the higher dose of EIF. Alternatively, the dose of L-NAME used in our experiments may have been insufficient to block completely the EIF-induced nitric oxide release. Prostacyclin, which is an arachidonic acid metabolite through cyclo-oxygenase, is considered to be another relaxing factor dependent on endothelial cells (Grygleweski *et al.*, 1991). In order to investigate whether prostacyclin was involved in EIF-induced relaxation, we also examined the effect of indomethacin, a cyclo-oxygenase inhibitor. At a concentration of 10 μM, indomethacin has been reported to inhibit the vasodilator effect of scoparone which is believed to act through prostacyclin (Huang *et al.*, 1992) and to inhibit propofol and thiopentone-induced cyclo-oxygenase metabolism (Park *et al.*, 1992). Our data demonstrated that the same concentration of indomethacin had no effect on EIF-induced relaxation, which excluded the involvement of prostacyclin at the highest dose of EIF used. The reasons for the differences in blockade of ACh and EIF-induced relaxation by L-NAME therefore remain to be elucidated.

Hallaq & Haupert (1989) have also isolated an endogenous substance which stimulates cardiac contractility. The isolation of endogenous substances from plasma of volume-expanded dogs (Gruber *et al.*, 1989), human plasma (Kelly *et al.*, 1985; Balzan *et al.*, 1986), human urine (Goto *et al.*, 1993) and other tissues (Ebara *et al.*, 1986) has also been reported. These substances or factors were demonstrated to share some pharmacological and immunological properties with ouabain (Hamlyn *et al.*, 1991) or digoxin (Haddy & Pamnani, 1983; Haddy, 1987). Although the factor we isolated from porcine heart left ventricle shares positive inotropic properties with these digitalis-like factors, our investigations demonstrated that EIF only weakly cross-reacts with digoxin anti-serum (Khatter *et al.*, 1991). Furthermore, EIF, which increases cardiac muscle contractility, lacks digitalis toxicity even at higher doses.

Our preliminary data from chemical and structural studies (unpublished data) suggest that EIF is a non-protein or non-peptide, small molecule (molecular weight less than 500) and a highly polar structure, which lacks the steroid structure present in digoxin, ouabain and most known digitalis-like compounds.

In summary, EIF induces relaxation of vascular smooth muscle contracted by PE. This effect is endothelium-dependent and is mainly through the stimulation of nitric oxide synthase in endothelial cells to release nitric oxide which relaxes smooth muscle cells. EIF does not change the basal tone of the tissue preparations. Prostacyclin is not involved in EIF-induced relaxation at the concentration of EIF used. Whether EIF acts via its own receptor to increase intracellular calcium concentration or via other pathways to activate nitric oxide synthase is not yet clear. The possibility that EIF may act through the muscarinic receptor has been excluded by the data presented in this paper.

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