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The inhibition of release of endothelium-derived relaxant factor by manoalide, a potent inhibitor of phospholipase A_2

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1 The inhibitory action of manoalide on vascular relaxation was characterized. Manoalide was a potent inhibitor of endothelium-dependent relaxations in the isolated aorta of the rabbit. Responses to acetylcholine (ACh), A23187 and substance P were reduced by manoalide in a dose-dependent manner whilst those to nitroglycerin were unaffected.

2 Repeated washing of manoalide-treated tissues did not restore the relaxant response to ACh, indicating an irreversible action of manoalide. Scanning electron microscopic studies revealed that the endothelium remained intact on manoalide-treated tissues.

3 Rabbit aortae from which the endothelium had been removed relaxed in response to perfusion with ACh when delivered via an upstream endothelium-bearing tissue, indicating release of an endothelium-derived relaxant factor (EDRF). Incubation of the tissue without endothelium with manoalide (100 nM; 30 min) or inclusion of manoalide in the superfusate at a point just distal to the endothelium bearing tissue did not reduce the relaxant potency of EDRF.

4 Contractile responses of the guinea-pig isolated ileum to ACh were not affected by manoalide and, furthermore, binding of [^{3}H]-quinuclidinyl benzilate to striatal membranes was not reduced by manoalide except at very high concentrations.

5 Manoalide therefore appears to inhibit vascular relaxation with a selectivity directed towards that mediated by EDRF. A direct antagonism of neither cholinoceptors nor EDRF receptors occurs and it is suggested that manoalide acts at a site within the endothelium to inhibit the synthesis and/or release of EDRF. Based upon these and previous data the possibility that EDRF is lipid-like or controlled by an arachidonic acid metabolite must continue to be considered.

Introduction

Endothelium-dependent relaxation responses have been well described for many agonists and in many preparations (see Berkowitz & Ohlstein, 1986 for review). Although the relaxant factor has not yet been identified, several drugs have been found to be effective in inhibiting the production and/or release of the factor (Griffith *et al.*, 1984). Such agents are putative inhibitors of various stages of arachidonic acid metabolism and have been shown to reduce the magnitude of endothelium-dependent relaxations. However, a firm relationship between a specific enzyme and the production of the relaxant factor has not been established. Exogenous arachidonic acid itself has been shown to induce a relaxation in vascular preparations which is reduced by removal of the endothelium (De Mey *et al.*, 1982). However, it is not known whether this phenomenon is due to a receptor-mediated event or to increased availability of substrate. In this regard, it is of interest that other unsaturated fatty acids also induce endothelium-dependent relaxations (see Furchgott , 1984).

Quinacrine and *p*-bromophenacyl bromide (BPB) are both inhibitors of phospholipase A_2 (PLA_2) and are also effective in reducing endothelium-dependent relaxations. The conclusions drawn from such studies may, however be tenuous since quinacrine is not effective against A23187-induced relaxations (Furchgott, 1984) and muscarinic blockade has been implied. Also, Furchgott (1983) noted that BPB frequently caused a widespread loss of endothelial cells which may itself produce the reduction of relaxation noted. Melittin on the other hand has received attention as an

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activator of PLA_2 and has been shown to induce endothelium-dependent relaxations (Forstermann & Neufang, 1985).

Shikano *et al.* (1986) have studied a range of Δ5-lipoxygenase inhibitors and showed that whilst these compounds were indeed effective in reducing the endothelium-dependent response there was no obvious correlation between the potency of inhibition of Δ5-lipoxygenase and the reduction in EDRF response. This finding must therefore question the mechanism of action of Δ5-lipoxygenase inhibitors on this response.

Peach *et al.* (1985) have speculated upon the involvement of cytochrome P450-oxygenase in the synthesis of EDRF, based upon the inhibitory actions of SK&F 525A. As discussed by Berkowitz & Ohlstein (1986), despite the almost complete inhibition of endothelium-dependent relaxation responses given by this drug, its actions may well be mediated by its calcium channel blocking activity (Lee & Berkowitz, 1976; Long & Stone, 1985).

Recent work by Murray *et al.* (1986) suggests that the EDRF may be a highly polar moiety since in the system described, EDRF-like activity was retained in the aqueous fraction and not in the organic phase. Fatty acid derivatives are routinely extracted into ethyl acetate prior to analysis. The result reported by Murray *et al.* (1986) therefore argues against the lipid hypothesis of EDRF.

Clearly, if the EDRF is derived from arachidonic acid the response to relaxing agents dependent on the endothelium will be sensitive to selective PLA_2 inhibitors. Manoalide (3,7-di(hydroxymethyl)-4-hydroxy-11-methyl(2,6,6-trimethylcyclohexenyl)-2,6-10-tridecatrianoic acid lactone) is a non-steroidal sesterterpenoid product of the sponge, *Luffariella variabilis*, which has been shown to possess anti-inflammatory actions. The structure is unrelated to other anti-inflammatory drugs and has been shown to be a potent inactivator of PLA_2 (Glaser & Jacobs, 1986). The actions of manoalide on endothelium-dependent relaxations were therefore investigated in order to address the question of the involvement of PLA_2 in the mediation of these responses and further characterize the nature of the EDRF.

Methods

Rabbit isolated aorta

Male New Zealand White rabbits (2–3.5 kg) were killed by cervical dislocation and exsanguinated. The aorta was quickly placed in oxygenated Krebs bicarbonate solution and cleaned of fat and connective tissue. The aorta was cut into rings 4 mm in length. The aortic rings were mounted on stainless steel hooks under 4 g resting tension in 25 ml isolated organ baths containing Krebs bicarbonate solution of the following composition (mM): NaCl 113, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2 and glucose 11.5. Indomethacin (10 μM) was routinely included in the Krebs solution. The organ bath solutions were maintained at 37°C and aerated with 95% O_2/5% CO_2. Tissues were allowed to equilibrate for 1 h before the experiments, with changes of solution every 20 min. Isometric tensions were recorded on a Beckman R-611 Dynograph recorder with Grass FTO3 transducers.

Tension was induced in aortic rings with phenylephrine (PE) (300 nM). Approximately equipotent relaxation responses to acetylcholine (ACh, 100 nM), A23187 (100 nM) or nitroglycerin (100 nM) were obtained by adding the relaxant to the organ bath solution upon attainment of a stable tone. Where manoalide was used, an incubation period of 30 min was allowed before further experimentation.

Cascade experiments

An aortic segment approximately 30 mm in length was cannulated for approximately 4 mm with polyethylene tubing and perfused with oxygenated Krebs solution at a rate of 9 ml min^{-1}. Care was taken with this preparation in order to maintain the integrity of the endothelium.

An aortic ring was prepared and the endothelium removed by gentle abrasion with a small wooden applicator. Effectiveness of this procedure was determined by the lack of a relaxant response to ACh (Furchgott & Zawadzki, 1980). The ring without endothelium was then mounted directly under the cannulated aorta and in the flow of the effluent. The temperature of the perfusates was maintained at 37°C by a circulating water bath. Tone was induced by the inclusion of PE (300 nM) in the medium and when tone was stable, ACh (1 μM) was also included. Following the responses to EDRF the ring without endothelium was taken down and incubated in manoalide (100 nM) for 30 min. After this period, the ring was remounted under the cannulated aorta, allowed to equilibrate for 30 min and again tested for responsiveness to EDRF as above. In some instances the experiment was performed as above with an additional perfusate included at the effluent from the cannulated aorta. The additional perfusate, also at 9 ml min^{-1}, was of either Krebs solution alone or including manoalide (100 nM).

Guinea-pig isolated ileum

Female guinea-pigs (200–300 g) were killed by cervical dislocation. The terminal ileum was removed and the luminal contents washed out with Krebs solution. The ileum was cut into 3 cm segments which

were tied at each end with silk thread and mounted in an isolated organ bath under 1 g tension, bathed in Krebs solution and maintained at 37°C. Responses to ACh (100 nM) were obtained by addition of the drug to the organ bath at 5 min intervals with washing of the tissue after 30 s exposure. Where manoalide was used, the drug was allowed to incubate with the tissue for at least 30 min before further experimentation.

[^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding

Rat brain striatal membranes were prepared from frozen tissues dissected from male rats. Tissues were stored at −80°C until used. Six striata (approximately 300 mg wet weight) were homogenized in 5 ml of ice cold 0.32 M sucrose with a glass homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatent transferred to 50 mM sodium potassium phosphate buffer (pH 7.4) to give a final membrane concentration of 1 to 1.5 mg wet weight tissue per ml. The membrane solution was rehomogenized on ice by polytron at setting number 7 for 60 s.

The binding assay was performed as described by Yamamura & Snyder (1974) in a total volume of 2 ml using 1 ml of the tissue preparation per assay tube. The [^{3}H]-QNB concentration used for the competition assay was 0.1 nM in 50 mM sodium potassium phosphate buffer (pH 7.4). Where manoalide was used, the tissue was pretreated with the drug for 20 min at 22°C before incubation with [^{3}H]-QNB. Incubation proceeded for 60 min at 37°C. After incubation, 5 ml of cold 50 mM phosphate buffer (pH 7.4) was added and the samples were rapidly filtered (<10 s) through Whatman GF/B filters. Filters were washed 3 times with 5 ml each of ice-cold buffer. Manoalide was tested at seven concentrations, performed in triplicate. Non-specific binding was assessed by inclusion of atropine (1 μM) and the IC_{50} of specifically bound ligand was determined by least squares regression analysis.

Chemicals

ACh, A23187, substance P, phenylephrine, indomethacin and atropine were purchased from Sigma. Nitroglycerin was obtained from Lilly pharmaceuticals. [^{3}H]-quinuclidinyl benzilate was purchased from New England Nuclear. Manoalide was a gift from Allergan Inc., Irvine, Ca. All other chemicals used were of reagent grade.

Results

The actions of manoalide were studied with respect to the relaxations induced by ACh, A23187, substance P (SP) and nitroglycerin (NG). In earlier experiments it was shown that relaxations due to ACh, A23187 and SP were abolished following gentle rubbing of the luminal surface of the vessel, whereas relaxations to NG were unaffected.

The inclusion of manoalide in the bathing medium did not affect the contraction to PE except at concentrations in excess of 1 μM, where a diminution of contraction was noted. Manoalide reduced in a dose-dependent manner the relaxations due to lower concentrations of ACh and A23187 which typically elicited control relaxations of approximately 70% of the induced tone (Figure 1). Relaxations to NG were unaffected by manoalide.

Endothelium-dependent relaxations to SP were also reduced by manoalide (Figure 2). Manoalide 30 nM considerably reduced the relaxation to SP over the range 0.1–10 nM with no apparent effect on the phenylephrine-induced tone. Relaxation responses to SP at concentrations up to 10 nM were abolished by manoalide (1 μM). This concentration of manoalide also caused a marked reduction in the phenylephrine-induced tone. IC_{50} values for manoalide inhibition of relaxation responses have been calculated and are presented in Table 1.

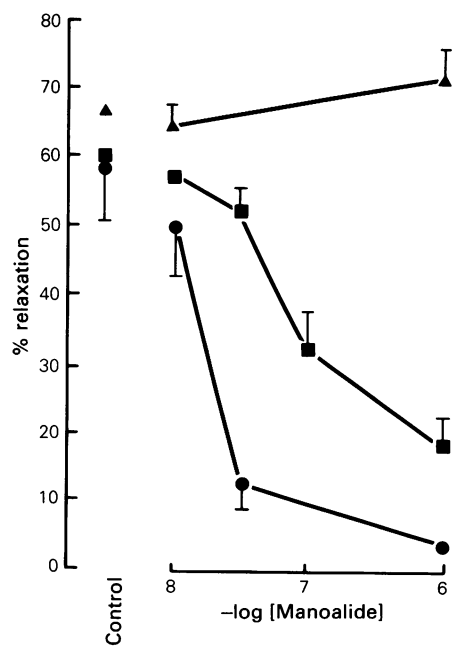

Figure 1 The effect of manoalide on vascular relaxation. Tone was induced by phenylephrine (PE) and relaxation induced by acetylcholine (100 nM; ●; $n = 4$), A23187 (100 nM; ■; $n = 3$) or nitroglycerin (100 nM; ▲; $n = 4$). Relaxation is expressed as a percentage of the PE-induced tone.

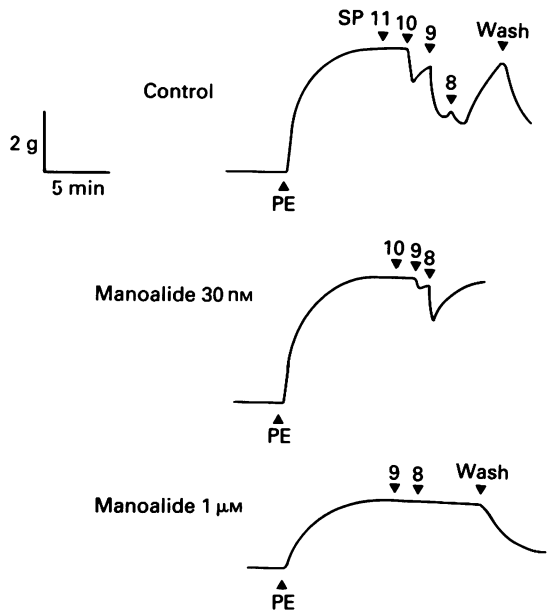

Figure 2 Relaxation responses to substance P (SP) in the rabbit aorta. The upper trace indicates the response in control tissues to SP (10^{-11}–10^{-8} M). Manoalide (30 nM) included in the bathing medium 30 min before the induction of tone reduced the relaxation due to SP (middle trace). The lower trace represents the response in the presence of 1 μM manoalide. Relaxations to SP up to 10^{-8} M were abolished and this concentration of manoalide also reduces the tone induced by phenylephrine (PE).

Table 1 IC_{50} values for manoalide inhibition of endothelium-dependent relaxant responses

Relaxant	*IC_{50}*	
Acetylcholine	22 ± 6 nM	(n = 4)
A23187	105 ± 27 nM	(n = 3)
Substance P	64 ± 18 nM	(n = 3)

Tone was induced with phenylephrine (300 nM) then relaxations were attempted with the above agents. Where manoalide was included in the organ bath an incubation of 30 min was allowed at each concentration used before further experimentation. Tabulated values are mean ± s.e.mean.

In some experiments where an inhibition of relaxation by 30 nM manoalide had been established, no restoration of relaxation responses was achieved even after 3 h of washing in manoalide-free solution (result not shown).

Histological studies with scanning electron microscopy revealed the luminal surface of manoalide-treated vessels (100 nM; 90 min) to be similar in endothelial integrity to control tissues (results not shown).

Guinea-pig isolated ileum

Application of ACh to the guinea-pig ileum evoked a contractile response. Manoalide (100 nM) had no effect on the contractile response to ACh (100 nM) whereas atropine (1 μM) inhibited the response by 25 ± 4% (n = 3).

[^{3}H]-QNB binding

Specific binding of [^{3}H]-QNB in the tissue was approximately 93%. Manoalide reduced specific binding of [^{3}H]-QNB only at concentrations above 5 μM. The IC_{50} values for displacement of [^{3}H]-QNB by manoalide or atropine were 16 μM and 3.2 nM respectively (Figure 3).

EDRF receptors

Tissues without endothelium were not relaxed by ACh. However, upon superfusion downstream of a perfused endothelium-bearing aorta, inclusion of ACh (1 μM) in the medium induced a relaxation of the bioassay ring. Pre-incubation of the bioassay ring in 100 nM manoalide for 30 min did not markedly reduce the relaxation response to released EDRF (Figure 4).

In two experiments, manoalide was perfused onto the tissue without endothelium from a source just

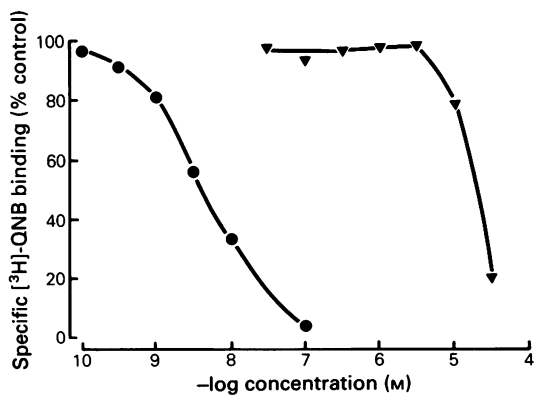

Figure 3 Inhibition of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to rat striatal membranes in the presence of atropine (●) or manoalide (▼). The results presented are the means of two experiments. Binding was unaffected by manoalide at concentrations below 10 μM.

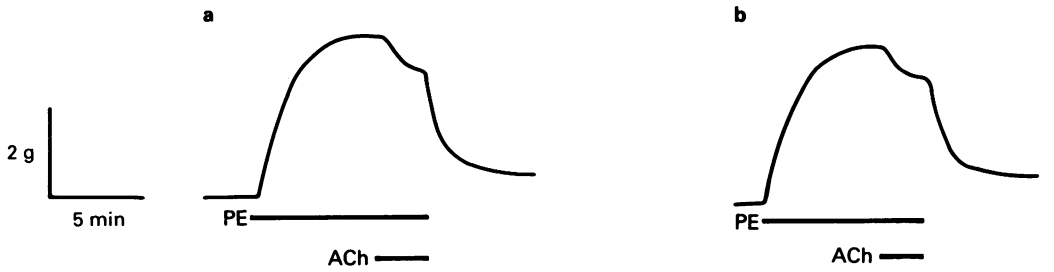

Figure 4 Aortic rings with endothelium removed did not relax to direct application of acetylcholine (ACh). However, in a perfusion system where an ACh containing solution initially passed through an endothelium bearing aorta, relaxation was observed (a). When the bioassay tissue (but not the endothelium bearing aorta) had been incubated in manoalide (100 nM; 30 min) a similar relaxation to ACh was again noted (b).

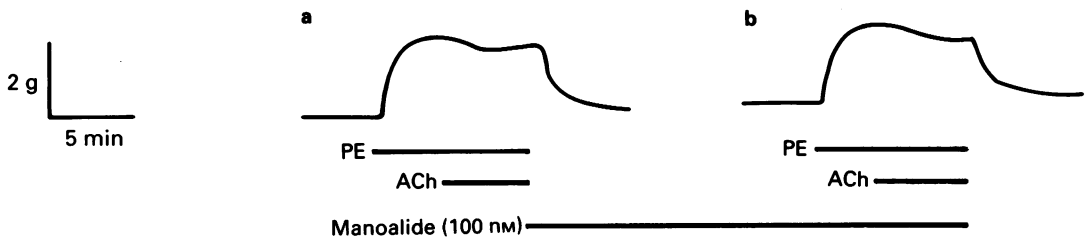

Figure 5 Tension responses of a 'recipient' aortic ring without endothelium Krebs solution alone (a) or containing manoalide (100 nM) was infused into the effluent just distal to an endothelium-intact aortic segment; the mixed solutions superfused a manoalide-pretreated aortic ring lacking endothelium. (a) Shows the typical response to administration of ACh through the perfusion system. The presence of manoalide (b) did not give any apparent reduction in the relaxation to ACh.

distal to the cannulated endothelium-intact aorta. The tissue without endothelium was in this case also pretreated with manoalide. The subsequent relaxation by EDRF released into a manoalide perfusion was also similar to the relaxation obtained when EDRF was released into Krebs solution alone (Figure 5). The relaxations obtained on the bioassay were small in size, presumably due to the large dilution of EDRF which occurs in the cascade. Such responses make it difficult to analyse data on a quantitative basis. However, the concentration of manoalide used (100 nM) profoundly inhibited relaxations due to ACh in the endothelium-intact ring. From this standpoint, given that no apparent reduction was noted on perfusion with manoalide, it is unlikely that manoalide inactivates the factor en passage to the bioassay.

Discussion

The phenomenon of endothelium-dependent relaxation was first demonstrated by Furchgott & Zawadzki (1980) and has been shown to be due to the release of a labile factor (Van de Voorde & Leusen, 1983). The half-life of EDRF is extended by superoxide dismutase (Vanhoutte & Rubanyi, 1985) and the relaxations due to the factor are reduced by anti-oxidants and also inhibitors of lipoxygenase and phospholipase A_2. Moncada *et al.* (1986) however, have cautioned that the anti-oxidants in particular are probable generators (or preservers) of superoxide ion which would be expected to inhibit the relaxant potency of the factor. This finding does not detract from the supposition that the EDRF is a fatty acid derivative since the polyunsaturated fatty acids are susceptible to free radical peroxidation and moreover, the decomposition of hydroperoxides have been reported to be catalysed by iron salts and transition metal complexes (see Halliwell & Gutteridge, 1985). By analogy, the EDRF is inactivated by ferrous ion (Gryglewski *et al.*, 1986) and also reduced haemoglobin (Martin *et al.*, 1985). The question of binding and enhanced inactivation of the factor must therefore be addressed in any study of inhibition of EDRF activity. Endothelium-dependent

relaxations to a variety of agents were inhibited by manoalide. Responses to nitroglycerin which similarly induces a relaxation associated with increased smooth muscle cyclic GMP (but independently of the endothelium) were unaffected. Since manoalide failed to reduce EDRF-induced relaxations when the compound was present in a perfusion system it is unlikely that the mechanism of action is due to binding of the factor en route to the bioassay. Similarly, this experiment also argues against antagonism of the EDRF at the smooth muscle.

In addition to activity against PLA_2, an inhibitory activity of manoalide on phospholipase C (PLC) may also occur (Bennett *et al.*, 1986). EDRF activity is not impaired by aspirin (Furchgott & Zawadski, 1980) even at the high concentration used to block prostaglandin H_2 synthetase. Bomalaski *et al.* (1986) have shown that PLC is potently inhibited by aspirin with an IC_{50} of approximately 1 μM. Clearly, as EDRF responses are not subject to aspirin inhibition it is unlikely that manoalide is acting in this instance on PLC to reduce endothelium-dependent relaxations.

Δ5-lipoxygenase inhibition by manoalide, as determined by the conversion of arachidonic acid to leukotriene B_4, was reported by De Vries *et al.* (1986) using human polymorphic neutrophils. Although the action on endothelium may be due to inhibition of Δ5-lipoxygenase rather than PLA_2 this mechanism would also invoke the mediation of a fatty acid substrate.

In contrast anti-inflammatory steroids, such as dexamethasone which induce the biosynthesis of a PLA_2 inhibitor (Flower & Blackwell, 1979) were without effect on EDRF production in cultured bovine aortic endothelial cells or on rat aortic rings following pretreatment *in vitro* (Milner *et al.*, 1986). Whilst Milner *et al.* (1986) measured a 40% decrease in PLA_2-stimulated arachidonic acid release it is not known whether this degree of inhibition would be sufficient to reduce EDRF production.

Wheeler *et al.* (1987) have described a calcium channel blocking activity of manoalide in mouse spleen cells. Both contractile responses (Bolton, 1979) and endothelium-dependent relaxations (Long & Stone, 1985) are known to be dependent upon extracellular calcium. It is noteworthy that contractions of the smooth muscle of this tissue were unaffected by manoalide at concentrations at the bioassay up to 50 nM (Figure 4) although relaxation responses were clearly inhibited (Figure 2). At the higher concentration (1 μM) of manoalide however, there is some evidence of a reduction in PE-induced tone (Figure 2) which might be due to calcium channel blockade. At this concentration of manoalide, the endothelium-dependent relaxations to SP and ACh were abolished and those to A23187 considerably reduced.

The possibility of an action of manoalide acting at muscarinic receptors was excluded by an analysis of [^{3}H]-QNB binding. The QNB ligand was not displaced by manoalide at concentrations that induced a blockade of endothelium-dependent relaxations. Furthermore, manoalide was effective in blocking endothelium-dependent relaxations to substance P (Table 1). Clearly, the action of manoalide is directed against endothelium-dependent relaxations in general and not against a specific receptor type.

There was evidence of a preferential inhibition of ACh-induced relaxation over A23187 responses which may imply the involvement of dissimilar cellular activation mechanisms. It has already been demonstrated that the half-life of the EDRF may differ between species (Forstermann *et al.*, 1984) indicating a difference in factor or metabolism. Consequently it is possible that manoalide is more potent versus ACh than A23187 if the structure(s) or state of the factor(s) alters with the releasing stimulus.

In conclusion, manoalide is shown to be a potent inhibitor of EDRF release and/or synthesis. Since manoalide is also effective in reducing arachidonic acid turnover it is proposed that this action results in decreased EDRF release or synthesis. The possibility that EDRF is lipid-like in nature or controlled by an arachidonic acid metabolite must continue to receive attention.

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