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Complex effects of *Gillichthys* urotensin II on rat aortic strips

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1 The aim of this study was to determine whether the fish neuropeptide, *Gillichthys* urotensin II (GUII), possesses significant biological activity on rat aortic strips.

2 On intact strips, pre-contracted by noradrenaline (100 nM), low concentrations (0.1–0.5 nM) of GUII produced relaxations, while higher concentrations (1–10 nM) caused further contraction. On strips rubbed to remove endothelial cells, relaxations were absent but contractile responses to higher concentrations of GUII remained.

3 GUII (0.2–10 nM) produced dose-related contractions of quiescent, intact aortic strips. These contractions consisted of two components, tonic and phasic, and were potentiated in rubbed strips and in the presence of the antioxidant drug hydroquinone (10 μM).

4 Mepacrine (40 μM) and *p*-bromophenacyl bromide (50 μM) completely abolished contractions to GUII, but indomethacin (10 μM) and nordihydro-guaiaretic acid (10 μM) were without effect.

5 The phasic, but not the tonic, component of the contractile response was inhibited by nitrendipine (200 nM), and was absent in bathing medium from which Ca^{2+} had been omitted. Addition of EGTA (2 mM) to Ca^{2+}-free bathing medium abolished the residual tonic component.

6 GUII-induced contractions were completely abolished by the calmodulin antagonists trifluoperazine (50 μM) and W-7 (30 μM).

7 It is concluded that GUII, previously considered devoid of significant activity on mammalian tissues, produces potent endothelium-dependent relaxations and endothelium-independent contractions of rat aorta, and possible mechanisms underlying each response are discussed.

Introduction

Urotensin II (UII) is a dodecapeptide neurohormone which is synthesized by, and released from, the caudal neurosecretory system of teleost fish, (Pearson *et al.*, 1985). The amino acid sequence, which differs slightly among piscine species, has been determined (Bern *et al.*, 1985). Although UII is known to produce smooth muscle contraction and osmoregulatory changes in fish (Bern *et al.*, 1985), there have been few reports of activity in mammalian species, with the consequence that UII has been considered devoid of significant biological activity in mammals (Bern & Lederis, 1978; MacCannell & Lederis, 1983). However, early experiments were carried out with partially purified UII from extracts of fish urophysis; more recently, clear biological activity in mammals has been obtained with a synthetic UII (GUII), having the amino acid sequence of the peptide found in the long-jawed mudsucker, *Gillichthys mirabilis* (Pearson *et al.*, 1980). An initial observation that GUII produced dose-related relaxations of the mouse anococcygeus muscle (Gibson *et al*, 1984) prompted further studies of possible actions in mammalian species. In anaesthetized rats, GUII both increased arterial pulse pressure and lowered mean arterial blood pressure (Gibson *et al.*, 1986). Pulse pressure was also increased in pithed rats, and pressor responses to sympathetic nerve stimulation and vasoconstrictor drugs were reduced (Gibson *et al.*, 1986). Clearly, these results suggested that GUII influenced vascular smooth muscle tone in the rat. However, previous studies of GUII on isolated blood vessels from mammals have given inconsistent results. Muramatsu *et al.* (1979) found that partially purified UII could both contract and relax rabbit aortic strips, although the relaxation was considered to result from oxidation of the noradrenaline used to raise muscle tone. In a subsequent study, UII was shown to be without effect on several isolated artery preparations from rat (Muramatsu *et al.*, 1981), but

more recently, contractile effects of UII have been demonstrated in rat aortic strips (Itoh *et al.*, 1986). Given this confusion, and in the light of the results with anaesthetized and pithed rats, the present study was undertaken to determine whether GUII does indeed affect tone of rat aortic strips, and if so, to investigate the underlying mechanisms.

Methods

Male rats (Wistar strain; 250–350 g) were killed by stunning and exsanguination. The thoracic aorta was cleared of surrounding tissue and excised from the aortic arch to the diaphragm. This length of aorta was then bisected, and two helically-cut strips prepared. These were mounted in glass organ baths (2 ml) containing Krebs-bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 and glucose 11.1) which was maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2. A resting tension of 1.5 g was placed on the tissue and changes in tension recorded by force-displacement transducers (Grass FTO3 or Biegestab K30) attached to a Graphtec Linearcorder Mark VII (WR 3101). Each preparation was allowed to equilibrate for 45 min before beginning the experiment. Some preparations, before being mounted in the organ bath, were rubbed with a cotton wool bud, moistened with Krebs solution, in order to remove the endothelial cell layer. Lack of a functional endothelium in such preparations was confirmed by the loss of relaxation responses to acetylcholine (1 μM), which produced large relaxations (> 70%) of noradrenaline (100 nM)-induced tone in intact preparations.

Drugs used during this study were: atropine sulphate (MacFarlan Smith), *p*-bromophenacyl bromide (Sigma), ethylene glycol-*bis* (β-amino ethyl ether)- N, N, N′, N′-tetra-acetic acid (EGTA; Sigma), indomethacin (Sigma), mepacrine dihydrochloride (Sigma), mepyramine maleate (May & Baker), methysergide (Sandoz), noradrenaline bitartrate (Sigma), nordihydroguaiaretic acid (Sigma), phentolamine mesylate (Ciba), trifluoperazine dihydrochloride (Sigma), urotensin II (synthetic *Gillichthys*; Peninsula), W-7 (N-6-aminohexyl-5-chloro-l-naphthalene sulphonamide; Sigma). All drugs were dissolved in saline, except *p*-bromophenacyl bromide, nitrendipine, and nordihydroguaiaretic acid which were made up as stock solutions in ethanol and then diluted to the appropriate concentration with saline; the concentrations of ethanol added to the organ bath did not influence tissue reactivity. Further, indomethacin was dissolved in 0.5% Na_2CO_3.

Statistical analysis was carried out by means of Student's *t* test.

Results

Effect of Gillichthys *urotensin II on pre-contracted strips*

Tone of rat aortic strips was raised by 100 nM noradrenaline (NA); in preliminary experiments this concentration was found to be the minimum necessary to obtain a steady, sustained rise in tone.

The effect of GUII on NA-induced tone is shown in Figure 1. Low concentrations (0.1–0.5 nM) produced dose-dependent relaxations, while at higher concentrations (1–10 nM) the relaxations became progressively smaller and were reversed to contractions. This pattern of activity was observed in 5 separate preparations (Figure 2a). To determine whether these responses were dependent upon an intact endothelium, the

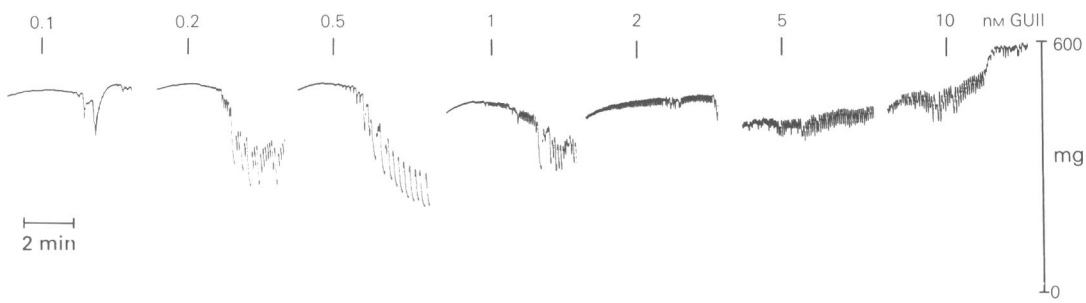

Figure 1 Responses of a rat intact aortic strip, pre-contracted by noradrenaline (100 nM), to increasing concentrations of *Gillichthys* urotensin II (GUII). The noradrenaline was added 5 min before GUII and the time interval between each trace was 30 min. Low concentrations of GUII caused relaxations, but these were reversed to further contractions at higher concentrations.

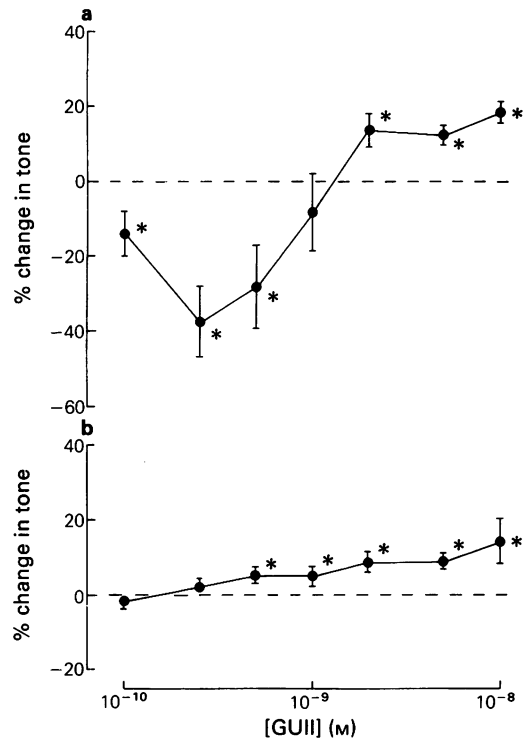

Figure 2 The percentage change of noradrenaline (100 nM)-induced tone in rat aortic strips produced by increasing concentrations of *Gillichthys* urotensin II (GUII). The protocol of the experiments was similar to that shown in Figure 1. (a) Shows the results from 5 intact strips, and (b) the results from 5 strips rubbed to remove the endothelium. Each point represents the mean and vertical lines show s.e. $*P < 0.05$, significant change in tone. In rubbed strips, GUII-induced relaxations were absent, but the contractile response remained.

experiments were repeated on rubbed strips; in these, GUII did not relax NA-induced tone, although the contractile effects of higher concentrations remained (Figure 2b).

Effect of Gillichthys *urotensin II on quiescent strips*

When added to quiescent aortic strips, GUII (0.2–10 nM) produced dose-related contractions (Figure 3). The most notable feature of these contractions was the appearance of two distinct components. At low concentrations of GUII there was a slow, steady (tonic) rise in tone, while at higher concentrations there was an additional rapid, oscillatory (phasic) contraction. As a result of these phasic contractions, the concentration range between the threshold and maximum response was narrow (Figures 3 and 4).

The experiments with strips pre-contracted with NA suggested that GUII might induce contraction of quiescent strips against a concurrent endothelium-dependent relaxation. This was confirmed in two ways. First, GUII-induced contractions were compared between intact and rubbed strips. The dose-response curve obtained from rubbed strips was slightly to the left of that from intact strips (Figure 4), although only at the lowest concentration studied (0.2 nM) was there a statistically significant potentiation of response ($P < 0.05$). Phasic and tonic components of contraction were observed in both intact and rubbed aortic strips. Secondly, it is known that endothelium-dependent relaxations of vascular smooth muscle are inhibited by antioxidant drugs, such as hydroquinone (Griffith *et al.*, 1984). On intact aortic strips, concentrations of GUII, which by themselves were just sub-threshold, produced contractions in the presence of hydroquinone (10 μM; Figure 5); in 4 such experiments, sub-threshold concentrations of GUII (0.2–1 nM) produced significant contractions

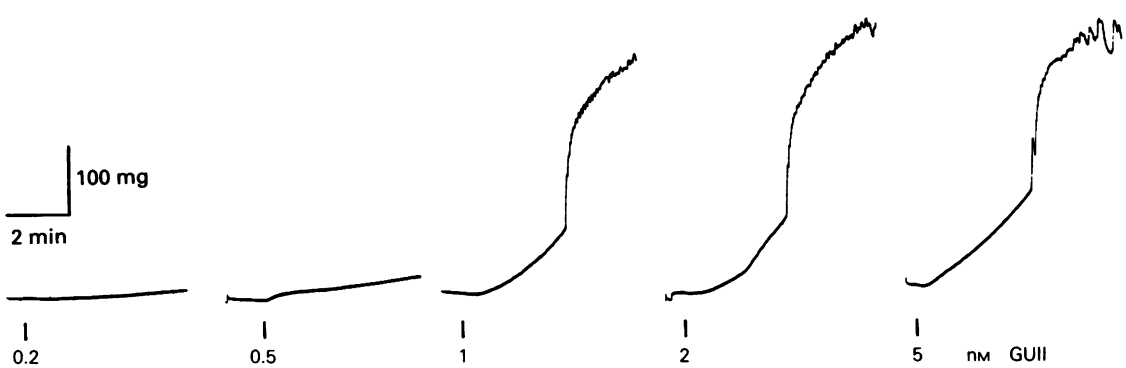

Figure 3 Responses of quiescent, intact aortic strips of the rat to increasing concentrations of *Gillichthys* urotensin II (GUII). The time interval between each trace was 30 min. GUII produced contractions consisting of two components, tonic and phasic.

(101 ± 8 mg; mean ± s.e.) in the presence of 10 μM hydroquinone.

The above experiments suggested that although GUII causes a weak endothelium-dependent relaxation of rat aortic strips the predominant effect is contraction. Therefore, the pharmacology of this contraction was investigated in more detail, and in all subsequent experiments rubbed strips were used.

Neurotransmitter antagonists

GUII (10 nM)-induced contractions were unaffected by phentolamine, atropine, mepyramine, or methysergide (all at 200 nM; Table 1).

Inhibitors of phospholipid metabolism

Mepacrine (40 μM) completely, but reversibly, abolished contractions of rat aorta to 10 nM GUII (Figure 6). Contractions were also abolished by *p*-bromophenacyl bromide (50 μM), but in this case the block was not reversed by washout, even up to 60 min after removal of the drug from the organ bath. Neither indomethacin nor nordihydroguaiaretic acid (both at 10 μM) affected contractions to GUII (Table 1).

Ca^{2+}-channel blocking drugs and Ca^{2+}-removal

The Ca^{2+}-channel blocking drug nitrendipine (0.1–1 μM) greatly reduced, but did not abolish, contractions to GUII (Table 1). In particular, nitrendipine selectively inhibited the phasic component of the contractile response (Figure 7a). In the presence of nitrendipine, the residual tonic contraction was abolished by mepacrine (40 μM). Although the tonic component returned on removal of mepacrine from the organ bath, the phasic responses did not recover after washout of nitrendipine (Figure 7a), even after 60 min.

Removal of extracellular Ca^{2+}, by simple omission from the Krebs solution, produced the same effect as nitrendipine; that is, loss of phasic, but retention of

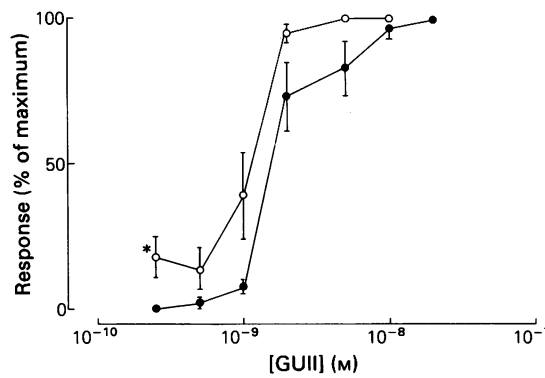

Figure 4 Dose-response curves of contractions induced by *Gillichthys* urotensin II (GUII) in intact (●) and rubbed (○) aortic strips from the rat. Each point represents the mean from 5 separate strips and vertical lines show s.e. There was no significant difference between the maximum tension increase produced by GUII in intact (218 ± 58 mg) and rubbed (295 ± 55 mg) strips. * $P < 0.05$, significantly greater than intact strip.

Table 1 Tension induced by *Gillichthys* urotensin II (GUII) in rubbed aortic strips of the rat before and after various drugs or procedures

Drug/procedure	*Response to 10 nM GUII* (mg tension) *Before*	*After*
Phentolamine (200 nM; 10 min)	332 ± 32	336 ± 27
Atropine (200 nM; 10 min)	316 ± 34	320 ± 28
Mepyramine (200 nM; 10 min)	308 ± 46	280 ± 32
Methysergide (200 nM; 10 min)	293 ± 29	306 ± 24
Mepacrine (40 μM; 10 min)	310 ± 44	0[a]
p-Bromophenacyl bromide (50 μM; 10 min)	326 ± 35	0[a]
Indomethacin (10 μM; 30 min)	272 ± 45	268 ± 41
Nordihydroguaiaretic acid (10 μM; 30 min)	282 ± 36	300 ± 44
Nitrendipine (200 nM; 10 min)	308 ± 49	55 ± 11[b]
Ca^{2+}-free Krebs (10 min)	270 ± 27	50 ± 4[b]
Ca^{2+}-free Krebs + EGTA (2 mM; 10 min)	304 ± 24	0[a]
Trifluoperazine (50 μM; 60 min)	282 ± 25	0[a]
W-7 (30 μM; 60 min)	308 ± 17	0[a]

Values are mean ± s.e. from 5 separate strips in each case. Drug concentrations and incubation times are given in parentheses.

[a] Drug/procedure completely blocked responses to GUII in all 5 strips.

[b] Represents selective block of phasic component of the contractile response ($P < 0.05$ compared with response before).

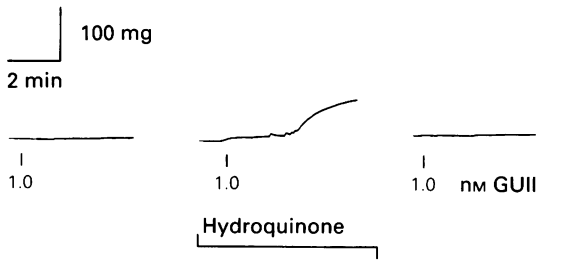

Figure 5 The effect of hydroquinone (10 μM) on the sensitivity of an intact aortic strip from the rat to *Gillichthys* urotensin II (GUII). Hydroquinone was added 10 min before GUII and the time interval between each trace was 30 min. In this preparation, 1 nM GUII was found to be just sub-threshold; however, in the presence of hydroquinone it caused a contraction.

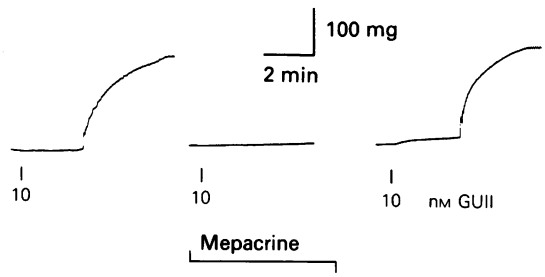

Figure 6 The effect of mepacrine (40 μM) on contractions of a rubbed aortic strip from the rat to *Gillichthys* urotensin II (GUII). Mepacrine was added 10 min before GUII and the time interval between traces was 30 min. Mepacrine completely abolished responses to GUII.

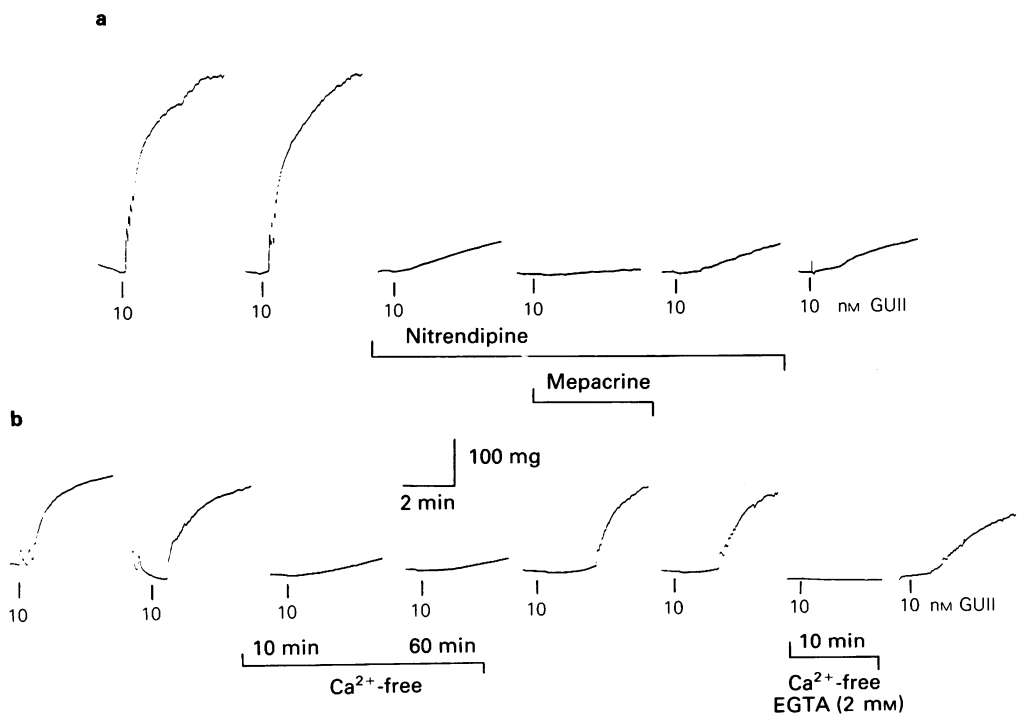

Figure 7 The effect of nitrendipine (a) and of Ca^{2+}-removal (b) on contractions of rat rubbed aortic strips to *Gillichthys* urotensin II (GUII). (a) Nitrendipine (200 nM) was added 10 min before GUII and the time interval between each trace was 30 min. In the presence of nitrendipine the phasic component of the contraction was inhibited, and the residual tonic component was blocked by further addition of mepacrine (40 μM). The tonic component recovered on removal of mepacrine. However, the phasic response did not recover on removal of nitrendipine. (b) The time interval between each trace was 30 min, except that it was 50 min between the two responses in Ca^{2+}-free medium. Simple omission of Ca^{2+} from the bathing medium (Ca^{2+}-free) resulted in loss of the phasic component of the contraction (i.e., similar to the effect of nitrendipine in (a)); phasic responses re-appeared when Ca^{2+} was added to the medium. Addition of EGTA (2 mM) to the Ca^{2+}-free bathing medium resulted in loss of both phasic and tonic components.

tonic contractions (Figure 7b). The residual tonic component persisted for up to 60 min in Ca^{2+}-free Krebs solution, and the phasic component returned on re-addition of Ca^{2+} to the medium. However, if EGTA (2 mM) was added to Ca^{2+}-free Krebs solution there was loss of both phasic and tonic components of the contractile response to GUII (Figure 7b; Table 1).

Calmodulin antagonists

The calmodulin antagonists trifluoperazine (50 μM) and W-7 (30 μM) completely abolished contractions of rat aorta to 10 nM GUII (Table 1).

Discussion

Clearly, the rat aorta is an example of a mammalian tissue on which GUII produces significant effects in low concentrations (see Larson *et al.*, 1985). The overall response is complex, being the resultant of two opposing actions, endothelium-dependent relaxation and endothelium-independent contraction. Contraction is, however, predominant since removal of the endothelium resulted in only slight potentiation of the contractile response to GUII. In essence, the results of this study agree with those of Muramatsu *et al.* (1979) using rabbit aorta, although the interpretation of results, based on current knowledge, would be slightly different. Muramatsu *et al.* (1979) showed that antioxidants prevented the relaxation of rat aorta induced by partially purified UII and proposed that UII caused oxidation of the drugs used to raise the tone of the preparation. However, it is likely that these relaxations, and those of rat aorta described in the present study, were due to the release of endothelium-derived relaxant factor (EDRF; Furchgott, 1983). The actions of EDRF are known to be blocked by antioxidants (Griffith *et al.*, 1984), and the antioxidant hydroquinone potentiated contractile responses of rat aorta to GUII, presumably by negating the opposing effect of released EDRF.

In anaesthetized or pithed rats, GUII both increased arterial pulse pressure and reduced pressor responses to drugs and nerve stimulation (Gibson *et al.*, 1986). The increased pulse pressure might be explained by the contraction of rat aorta demonstrated in this study. The large arteries, by virtue of their elastic properties, act as a damper on the oscillations of pressure that occur during the cardiac cycle. Therefore, any procedure which reduces their elasticity, such as increased tone, also reduces the damping action, leading to increased pulse pressure (Levenson *et al.*, 1985). Indeed, Itoh *et al.* (1986) have suggested that the contractile effects of UII are not confined to the aorta but extend to other large arteries in the rat. The second, vasodepressor, effect of GUII in rats cannot be explained simply in terms of aortic relaxation, but it is possible that a similar release of EDRF in more peripheral vascular beds could be involved.

The nature of the receptor on which GUII acts to induce aortic contraction has yet to be determined. Experiments with antagonists ruled out muscarinic acetylcholine receptors, α-adrenoceptors, histamine (H_1)-receptors, and 5-hydroxytryptamine receptors. GUII shows partial sequence homology, and shares some common actions, with somatostatin (Bern *et al.*, 1985). However, preliminary studies suggest that aortic contraction to GUII is not mediated via somatostatin receptors, since somatostatin (up to 1 μM) does not cause contraction of rat aortic strips (A. Gibson, unpublished observation).

Although the nature of the receptor remains obscure, the experiments did give some insight into the mechanisms underlying the contractile response to GUII. Both mepacrine and *p*-bromophenacyl bromide completely blocked the contraction. Both agents have been used as inhibitors of phospholipase A_2 (Vargaftig & Dao Hai, 1972; Volwerk *et al.*, 1974; Flower & Blackwell, 1976; Vallee *et al.*, 1979; Yamamoto *et al.*, 1982) to prevent liberation of arachidonic acid with subsequent production of its active metabolites. However, GUII-induced contractions were unaffected by cyclo-oxygenase (indomethacin) or lipoxygenase (nordihydroguaiaretic acid) inhibitors suggesting that products of arachidonate metabolism utilising these enzymes were not involved. Recent evidence has shown that both mepacrine and *p*-bromophenacyl bromide have a wider range of inhibitory effects on phospholipid metabolism, blocking both phospholipase A_2 and phospholipase C (Kyger & Franson, 1984); indeed, mepacrine is thought to act by binding to the phospholipid substrates rather than to inhibit specific enzymes (Hofmann *et al.*, 1982). Despite this non-selectivity, the complete inhibition observed in the presence of each drug strongly indicates that an early step in the receptor-contraction coupling process activated by GUII involves increased phospholipid turnover. The resulting contraction consisted of two components, tonic and phasic, and evidence was obtained that separate Ca^{2+} pools, and Ca^{2+} mobilization processes, are involved in each. First, the phasic contractions were prevented by nitrendipine and by simple omission of Ca^{2+} from the bathing medium, suggesting that this component results from movement of free extracellular Ca^{2+} (van Breemen *et al.*, 1982) through nitrendipine-sensitive voltage operated channels (Bolton, 1979). The tonic contraction was nitrendipine-insensitive and persisted in medium from which Ca^{2+} had been omitted, but when EGTA was added to Ca^{2+}-free medium the tonic response to GUII disappeared. This indicates that the tonic response also utilizes extracellular Ca^{2+}, but this pool is less exchan-

geable than that involved in the phasic contractions, and is possibly that bound to the external surface of the plasma membrane (van Breemen *et al.*, 1982). There is evidence that some agents may contract smooth muscle, in part, via a Ca^{2+}-independent mechanism (Casteels *et al.*, 1981; Ashoori *et al.*, 1985). However, GUII-induced contractions of the rat aorta appear to be wholly Ca^{2+}-dependent since they were totally inhibited in Ca^{2+}-free, EGTA-containing Krebs solution and by the calmodulin antagonists trifluoperazine and W-7.

Finally, the detection of potent biological activity of GUII on rat aortic strips has important implications. First, it confirms that the previously held view that UII is devoid of activity in mammals is incorrect and, therefore, that further studies with UII in other mammalian systems may prove fruitful (Larson *et al.*, 1985; Bern *et al.*, 1985). Secondly, the rat aorta might provide a useful bioassay preparation for the development of GUII antagonists. Such drugs would be of use in classifying the mammalian receptors (and in identifying the endogenous ligand), as well as in the elucidation of the physiological roles of UII in fish (Bern *et al.*, 1985).

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