Journal of Physiology (1990), **437**, *pp.* 543–561
With 8 figures
Printed in Great Britain

GTP AND NORADRENALINE-INDUCED FORCE IN ISOLATED TOXIN-PERMEABILIZED RAT ANOCOCCYGEUS AND GUINEA-PIG PORTAL VEIN

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(*Received 22 June 1990*)

SUMMARY

1. Strips of smooth muscle from rat anococcygeus and guinea-pig portal vein were treated with solutions containing crude α-toxin from the bacterium *Staphylococcus aureus*. This rendered the surface membrane permeable to small molecular weight substances, but left functional sarcolemmal adrenoceptors. Tension measurements from these preparations were used to investigate the effects of guanosine-5′-triphosphate (GTP) on the noradrenaline-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) of the smooth muscle of rat anococcygeus and guinea-pig portal vein.

2. Under conditions of low Ca^{2+} buffering (0·2 mM-EGTA), applying a maximal dose of noradrenaline (30 μM) to a toxin-permeabilized strip of anococcygeus muscle and longitudinal muscle of guinea-pig portal vein caused a transient contracture. Subsequent exposures to noradrenaline resulted in progressively smaller contractures. However, the rate of decline in the size of the noradrenaline-induced contracture was greater in rat anococcygeus muscle than in guinea-pig portal vein preparations. The decline in the size of the contracture in toxin-permeabilized anococcygeus muscle was not due to a fall in the Ca^{2+} content of the SR or a reduced Ca^{2+} release from the SR in response to *myo*-inositol 1,4,5-trisphosphate (IP_3).

3. The tension transients due to noradrenaline were enhanced and maintained in the presence of 100 μM-GTP in toxin-permeabilized guinea-pig portal vein. Addition of 100 μM-GTP caused a transient contracture in permeabilized rat anococcygeus muscle and only promoted the next noradrenaline response, thereafter the amplitude of the contractures decayed to zero.

4. Addition of guanosine-5′-*O*-(2 thiodiphosphate) (GDP-β-S, 100 μM) would be expected to cause a reversible reduction of the noradrenaline response by binding to the intermediary G-protein. This was observed in toxin-permeabilized portal vein, but in rat anococcygeus muscle, GDP-β-S caused slowing of the response to noradrenaline, thereafter the response to noradrenaline was absent. The noradrenaline response did not recover when GDP-β-S was removed.

5. The non-metabolizable form of GTP, guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S, 100 μM), caused a transient contracture in both toxin-permeabilized rat anococcygeus muscle and guinea-pig portal vein. In both these tissues, the addition of GTP-γ-S resulted in the irreversible inhibition of the response to noradrenaline.

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6. In the presence of a high concentration (10 mM) of the Ca^{2+} buffer EGTA, GTP (100 μM) and noradrenaline (30 μM) increased Ca^{2+}-activated force in both tissues. In guinea-pig portal vein preparations, the response due to GTP was small in comparison to the response due to noradrenaline. In rat anococcygeus muscle, the GTP response was dominant, the noradrenaline response being small in comparison.

7. In conclusion, marked differences exist between the responses of toxin-permeabilized smooth muscle from guinea-pig portal vein and rat anococcygeus to noradrenaline and GTP. Those effects observed in 0·2 mM-EGTA on guinea-pig portal vein may be interpreted as resulting from a G-protein-mediated α_1-adrenergic response. However, in rat anococcygeus preparations the α_1-adrenergic response was altered by additional factors that eventually abolished responses to noradrenaline.

INTRODUCTION

Two ways noradrenaline can affect smooth muscle directly are via the binding of noradrenaline to α- and β-forms of the adrenoreceptors. The α-adrenoceptor-mediated responses have been further divided into α_1- and α_2-forms (reviewed by McGrath, Brown & Wilson, 1989). The intracellular mechanism behind the α_1-adrenergic response is thought to involve the activation of phospholipase C and the production of *myo*-inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (reviewed by Minneman, 1988). IP_3 has been shown to trigger the release of Ca^{2+} from the sarcoplasmic reticulum (SR) in smooth muscle (Walker, Somlyo, Goldman, Somlyo & Trentham, 1987). DAG is thought to activate protein kinase-C (Nishizuka, 1984) leading to an increase in the sensitivity of the myofilaments to Ca^{2+} (Nishimura, Klober & van Breemen, 1988). Linking the α_1-receptor and phospholipase C is a membrane-bound G-protein. It is thought that α_1-adrenoceptor activation may cause IP_3 synthesis only if guanosine-5′-triphosphate (GTP) is bound to the G-protein (Michaelis–Menten constant, $K_m = 0{\cdot}75$ μM). However, GTP levels are not thought to modulate G-protein activity in intact cells since normal intracellular levels of GTP lie in the range 0·1–0·3 mM (Gill, Mullaney & Ghosh, 1988).

Recently, it has been shown that GTP and its non-metabolizable form, guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) can cause release of Ca^{2+} from the SR of permeabilized smooth muscle cells (cultured smooth muscle, Chueh, Mullaney, Ghosh, Zachary & Gill, 1987; rabbit main pulmonary artery, Kobayashi, Somlyo & Somlyo, 1988). A similar effect is seen in the endoplasmic reticulum (ER) of cultured nerve cells (Gill, Ueda, Chueh & Noel, 1986). Kobayashi *et al.* (1988) further showed that most of this effect was via GTP-stimulated IP_3 release. However, they saw a small IP_3-independent component of the tension transient. An additional effect of GTP was recently reported by Saida, Twort & van Breemen (1988). Their results suggest that IP_3 cannot release Ca^{2+} from the SR of saponin-permeabilized smooth muscle from rabbit mesenteric artery unless GTP is present.

α-Toxin from *Staphylococcus aureus* has been found to permeabilize the sarcolemma of smooth muscle (Nishimura *et al.* 1988; Kitazawa, Kobayashi, Horiuti, Somlyo & Smolyo, 1989; Smith, Crichton, McGrath & Miller, 1990). Use of α-toxin has two major advantages over previous treatments. Firstly, the holes created are small enough to prevent the loss of cytosolic proteins. Secondly, agonists can still trigger release of Ca^{2+} from the SR indicating that membrane-bound receptors remain intact.

This technique was used to study the adrenergic response in a visceral smooth muscle (rat anococcygeus) and a vascular smooth muscle (guinea-pig portal vein longitudinal muscle). Under the conditions used in the experiments, noradrenaline produced effects due to α_1-adrenergic stimulation. Neither tissue is thought to exhibit significant β-adrenergic effects under these conditions (Gillespie, 1980; Patel, Bose & Greenway, 1981). Noradrenaline was applied repetitively to toxin-permeabilized smooth muscle in the presence and absence of GTP. Important differences were discovered between responses from rat anococcygeus muscle and guinea-pig portal vein. Preliminary results have been reported to The Physiological Society (Crichton, Smith, McGrath & Miller, 1990; Smith *et al.* 1990).

METHODS

Wistar rats and Dunkin–Hartley guinea-pigs weighing 250–300 g were killed by a blow to the head followed by exsanguination. The anococcygeus muscle was removed from a rat, placed in Tyrode solution and strips 50–100 μm in diameter and 1–2 mm long were dissected from the tissue. The anterior section of the portal vein was removed from a guinea-pig, and thin bundles of longitudinal muscle (100 μm wide) were dissected from the wall of the vessel.

A muscle strip was attached between a force transducer (Akers AE875) and a fixed point by means of snares. This arrangement was used to hold the strip of smooth muscle in one of a series of chambers containing either 4·6 or 0·96 ml of fluid. The chambers were switched under microcomputer control to effect the solution change. An initial resting tension of 0·5 mN was applied to anococcygeus preparations prior to permeabilization with toxin. A smaller resting tension (0·1 mN) was applied to portal vein preparations. All diagrams show the active tension generated by the preparation. Tensions from separate experiments were averaged and expressed as means $\pm$ 1 S.D.

Solution composition

The Ca^{2+} buffer EGTA was used to control $[Ca^{2+}]$. The solutions contained ATP and phosphocreatine (PCr) to support contraction of the permeabilized muscle. Experiments were carried out at room temperature (20–22 °C). Solution compositions are listed in Table 1.

A range of $[Ca^{2+}]$ was obtained by mixing solutions A and B in differing proportions. A similar range of $[Ca^{2+}]$ was obtained in solution C (0·2 mM-EGTA) by adding calcium chloride (1 M titration standard, BDH). The equilibrium concentrations of metal ions were calculated using a computer program with the affinity constants for H^+, Ca^{2+} and Mg^{2+} binding to EGTA, taken from Smith & Miller (1984). The affinity constants used for ATP and PCr were those quoted by Fabiato & Fabiato (1979). Corrections for ionic strength, details of pH measurement, allowance for Ca^{2+} contamination, EGTA purity and the principles of the calculations are detailed elsewhere (Smith & Miller, 1984). GTP was purchased from Sigma. Guanosine-5′-*O*-(2-thiodiphosphate), (GDP-β-S); GTP-γ-S and IP_3 were purchased from Calbiochem.

Toxin-permeabilizing procedure

Figure 1 shows the protocol used to permeabilize the strips of smooth muscle. The strip of anococcygeus was initially exposed to 30 μM-noradrenaline while in Tyrode solution. This resulted in a contracture with a phasic and tonic component; removal of noradrenaline allowed tension to fall to baseline levels. The preparation was then placed in a mock intracellular solution (Solution C, Table 1) with a $[Ca^{2+}]$ of 63·5 μM which caused a phasic contracture. After 10 min in this solution the tension had declined to approximately half of the peak value. The introduction of crude α-toxin from *Staphylococcus aureus* (2 mg/ml) caused tension to slowly rise, eventually reaching a plateau after 10 min. The rise of tension presumably reflects the generation of force as the Ca^{2+} gained access to the contractile machinery through pores created by the action of the toxin. Once the tension had reached a plateau the toxin was removed with little change in the tension level. Lowering the $[Ca^{2+}]$ to 0·08 μM caused a rapid relaxation. Subsequent addition of caffeine or noradrenaline caused a transient contracture. Panel *B* of Fig. 1 shows the response of another strip of anococcygeus muscle exposed to a similar protocol, but without the toxin. Under these

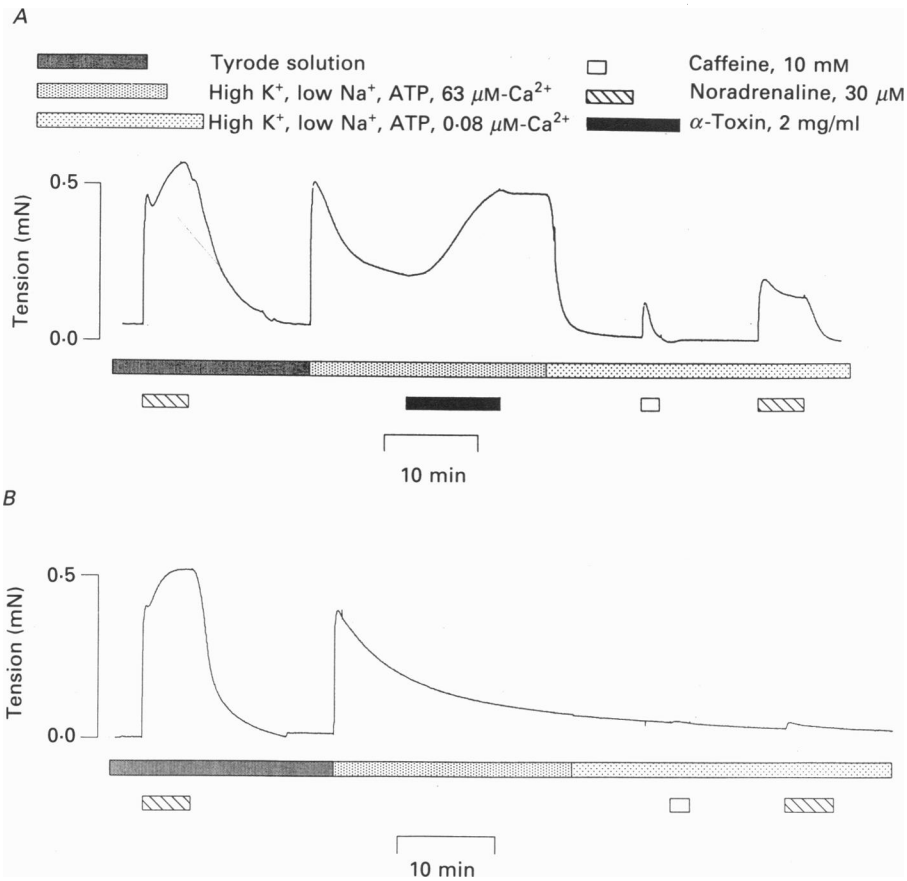

Fig. 1. Solution protocol used to permeabilize smooth muscle. Both panels show tension records from strips of rat anococcygeus muscle. The solution changes are indicated by the bars below each tension record. Panel *A*, the solution changes included the addition of crude α-toxin (2 mg/ml). Panel *B*, α-toxin was omitted from the bathing solution.

TABLE 1. Composition of solutions (in mM except where stated)

Solution	K^+	Na^+	Total Mg^{2+}	Total Ca^{2+}	Free Ca^{2+} (μM)	EGTA	$CH_3SO_3^-$	Cl^-	ATP	PCr	HEPES
A	120	40	7·0	10·0	31·6	10·0	100	14	5	15	25
B	120	40	7·7	0·02*	4×10^{-4}	10·0	100	14	5	15	25
C	120	40	7·0	0·02*	0·03	0·2	120	14	5	15	25
E(Tyrode)	5	120	1·0	2·0				131			5

Solutions A–C were pH 7·1; free Mg^{2+} was 2·3 mM; solution E was pH 7·4. *Total $[Ca^{2+}]$ estimated from previous measurements of Ca^{2+} contamination. ATP, PCr, EGTA, methylsulphonic acid (CH_4SO_3) and HEPES were purchased from Sigma.

circumstances lowering $[Ca^{2+}]$ had little effect, and neither caffeine nor noradrenaline caused a significant response compared to panel *A*. Thus with the protocol shown in panel *A* of Fig. 1, the progress of the action of the toxin can be monitored.

Caffeine causes the rapid release of Ca^{2+} from the SR of smooth, skeletal and cardiac muscle, this

is responsible for the tension transient seen on addition of 10 mM-caffeine in permeabilized preparations in the presence of low concentrations of EGTA. An additional effect of caffeine is the inhibition of the intracellular enzyme phosphodiesterase and the consequent accumulation of cyclic AMP, which acts on the myofilaments in smooth muscle to reduce Ca^{2+}-activated force (Meisheri & Rüegg, 1983). Caffeine does have a relaxant action in toxin-treated smooth muscle (C. A. Crichton & G. L. Smith, unpublished results) and this may affect the amplitude of the tension transient seen in caffeine. This effect prevents a quantitative comparison of the amplitude of the caffeine-induced contraction with other means of releasing SR Ca^{2+}. However, the effect of caffeine is reversible and does not prevent its use to assess changes in the Ca^{2+} content of the SR. The size of the caffeine-induced contraction was sensitive to the duration of the intervening period and the $[Ca^{2+}]$ in the bathing medium. But GTP, which radically affects the size of the noradrenaline-induced contraction, did not significantly affect the size of the caffeine-induced contraction. In this study, trains of caffeine-induced contractures of uniform size could be obtained for periods of up to 3 h if the time between contractures was kept constant (e.g. 10 min).

RESULTS

The effects of 100 μM-GTP on the noradrenaline response

Rat anococcygeus muscle

Strips of anococcygeus muscle were exposed to repeated applications of 10 mM-caffeine in the presence of 0·08 μM-Ca^{2+} (0·2 mM-EGTA). Provided the period of time between applications was kept constant (in this instance 10 min), transient contractures of uniform height were obtained, indicating that during each 10 min loading period the SR had accumulated a constant amount of Ca^{2+}. After caffeine contractures of uniform size had been achieved, the permeabilized muscle was exposed to 30 μM-noradrenaline (Fig. 2*A*). This lead to a transient contracture with a slower time course than the previous caffeine contractures. The average amplitude of the contracture was 0·12 mN (±0·043, $n = 17$) which represents 10% of maximal Ca^{2+}-activated tension. This response to noradrenaline is thought to be due to the activation of α_1-receptors in the sarcolemma, initiating the synthesis and release of IP_3, which in turn activates a transient release of Ca^{2+} from the SR. Subsequent application of noradrenaline caused a smaller contracture which was on average only 40·4% (±20·0%, $n = 9$) of the first contracture. After three contractures, the amplitude was reduced to 27·1% (±11·5%, $n = 9$) of the first noradrenaline response. To stimulate G-protein activity, GTP (100 μM) was added for the period indicated by the filled bar. GTP caused a transient contracture which was on average 64% (±14%, $n = 3$) of the first noradrenaline response. After relaxation, the subsequent response due to noradrenaline was enhanced to 67% (±24%, $n = 3$) of the first noradrenaline response, but the decay of the noradrenaline response continued, and after two further applications of noradrenaline the amplitude was profoundly reduced. Removal of GTP did not restore the size of the contracture. Instead, the amplitude continued to fall until only a small tonic component was visible. This form of the response differs from the results observed when GTP was added after the first exposure to noradrenaline shown in Fig. 2*B*. Under these circumstances, GTP produced a contracture which was of equal size or sometimes larger than the first noradrenaline response (by 114±22%, $n = 6$).

Unlike Fig. 2*A*, the first noradrenaline response in the presence of GTP was not larger than the previous noradrenaline response. The average amplitude was

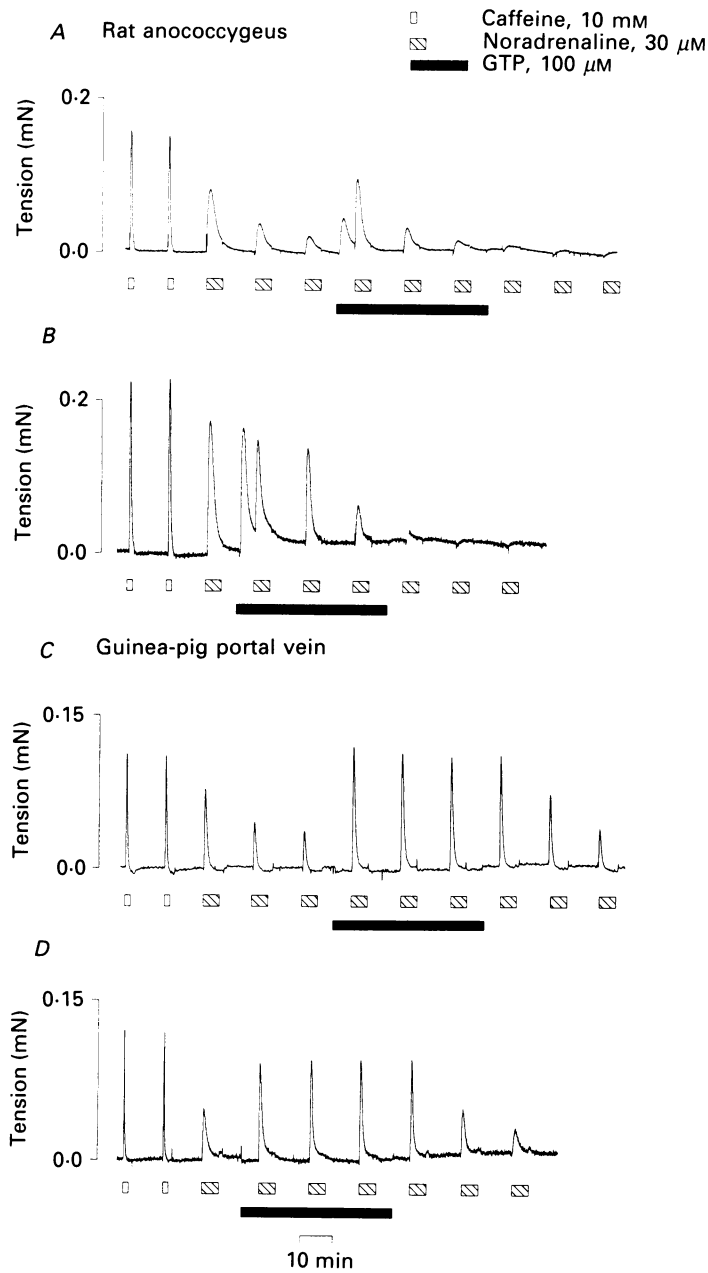

Fig. 2. The effect of 100 μM-GTP on the response to noradrenaline in permeabilized rat anococcygeus muscle (panels *A* and *B*) and guinea-pig portal vein (panels *C* and *D*). Addition of caffeine, noradrenaline and GTP are indicated by the bars below each tension record. Panels *A* and *C*, GTP was added after three noradrenaline responses. Panels *B* and *D*, GTP was added after one noradrenaline response.

73·1 % ($\pm$20 %, $n = 9$) which is significantly larger than the average second noradrenaline response in the absence of GTP (40·4 $\pm$ 20·0 %, $n = 9$) indicating that GTP can temporarily promote the size of the noradrenaline response.

Longitudinal muscle from guinea-pig portal vein

When similar protocols were applied to permeabilized strips of smooth muscle from guinea-pig portal vein the results contrasted markedly with those described above. In this preparation, the size of the first noradrenaline response was on average

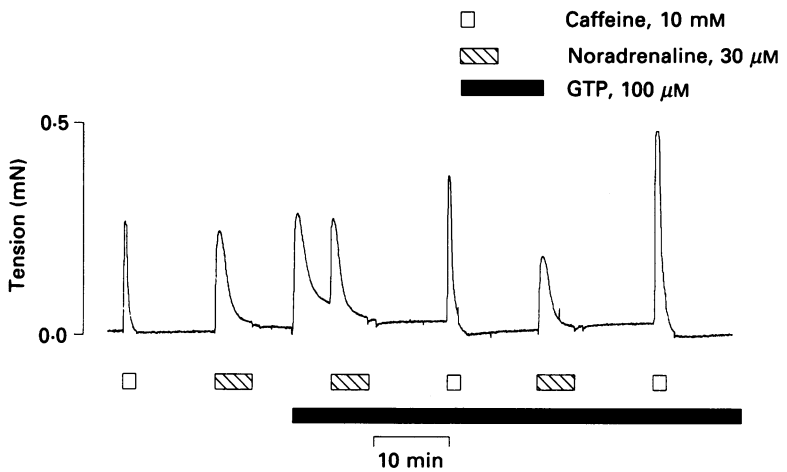

Fig. 3. The effect of noradrenaline and GTP on the caffeine responses in permeabilized rat anococcygeus muscle. Addition of caffeine, noradrenaline and GTP are indicated by the bars below the tension record.

0·09 mN (±0·052, $n = 12$), which represented 36% of maximal Ca^{2+}-activated force. As shown in Fig. 2*C*, the rate of decline in the size of the noradrenaline contracture (to 71±11%, $n = 9$ on the second and 58·5±13·2%, $n = 9$ on the third contracture is less than that observed in rat anococcygeus muscle ($P < 0·001$). Also, 100 μM-GTP did not cause a transient contracture, and all subsequent responses to noradrenaline were enhanced (142±39% of the first noradrenaline response, $n = 3$). Unlike the results from rat anococcygeus muscle, this increase in the size of the response to noradrenaline was maintained in the presence of GTP. Figure 2*D* indicates that GTP enhanced the noradrenaline response, even if it was added after the first noradrenaline response (by 148±30%, $n = 3$) to give a sustained amplitude that is similar to that seen after GTP in Fig. 2*C*.

Comparison of the effects of noradrenaline, caffeine and IP_3 on permeabilized rat anococcygeus muscle

Caffeine

The decrease in the size of the response to noradrenaline may result from a fall in the Ca^{2+} content of the SR. To test this hypothesis, caffeine (10 mM) was used to assess the Ca^{2+} content of the SR. Figure 3 illustrates a typical experiment in which caffeine was included in the protocol. The caffeine response on the left-hand side was obtained by repeatedly applying caffeine between 10 min loading periods. The amplitude of the noradrenaline contracture declined, while the accompanying caffeine contractures increased in size. This result indicates that the reduced response

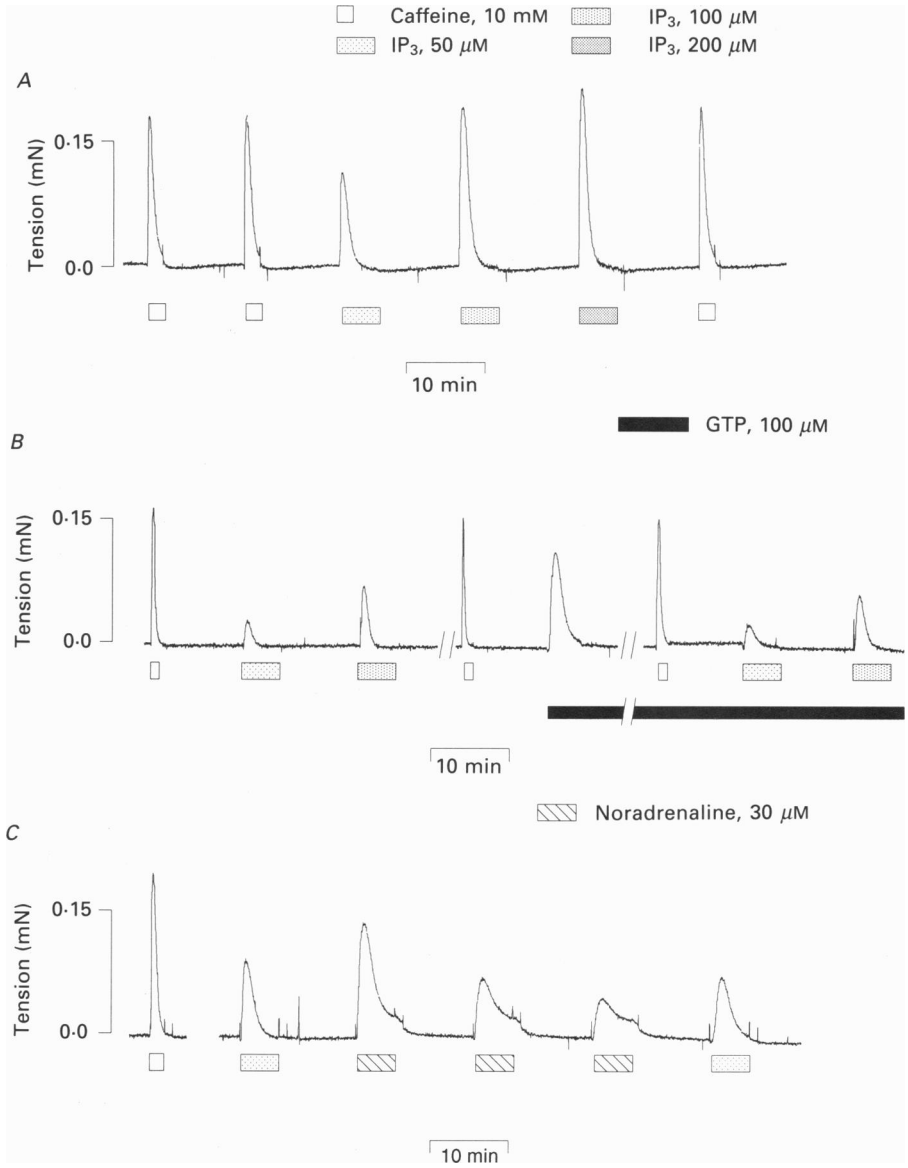

Fig. 4. The effect of noradrenaline and GTP on the IP_3-induced response in rat anococcygeus muscle. The addition of caffeine, noradrenaline, GTP and IP_3 are indicated by the bars below each tension record. Panel *A*, the effect of increasing IP_3 concentration on the tension response. Panel *B*, the effect of GTP on the IP_3-induced contraction. Panel *C*, comparison of the IP_3 response before and after three responses to noradrenaline.

to noradrenaline is not due to a fall in the Ca^{2+} content of the SR, and that the gradual rise in amplitude of the caffeine response may be the result of the increased period available for the Ca^{2+} loading of the SR.

myo-Inositol 1, 4, 5, trisphosphate (IP)$_3$

Another explanation for the decline of the noradrenaline contractures seen in rat anococcygeus muscle may be a reduction in the sensitivity of the SR to IP_3. Figure 4*A* shows that 50, 100 and 200 μM-IP_3 gave transient contractures. These contractures indicate the sensitivity of the preparation to externally applied IP_3. As shown in Fig. 4*B*, the presence of 100 μM-GTP did not alter the responses to IP_3. Figure 4*C* shows that after the decline in the response to noradrenaline, 50 μM-IP_3 produced a contracture of similar amplitude to the initial response to IP_3. Both experiments indicate that the fall in the size of the response to noradrenaline is not caused by a reduced ability of IP_3 to release Ca^{2+} from the SR. Instead, the smaller response may be due to a reduction in the amount of noradrenaline-triggered IP_3 synthesis.

The effects of 1 mM-GTP on the noradrenaline response

The absence of a response to GTP in permeabilized guinea-pig portal vein may reflect a relatively low sensitivity of the G-proteins in these preparations to GTP. To test this hypothesis, experiments were carried out using 1 mM-GTP. The contracture induced by 1 mM-GTP in rat anococcygeus was 116% ($\pm$43%; $n = 3$) of the first noradrenaline response. This was not significantly different to that observed with 100 μM-GTP. Similarly, the response of guinea-pig portal vein to 1 mM-GTP was not significantly different from that obtained after 100 μM-GTP (results not shown).

The effects of GTP-γ-S on the noradrenaline response

The response of the preparation to GTP-γ-S is shown in Fig. 5. As shown in panel *A*, the addition of 100 μM-GTP-γ-S resulted in a large transient contracture. This treatment abolished the response to noradrenaline, which did not recover on removal of GTP-γ-S. The size of the transient was similar when 10 μM-GTP-γ-S was used (not shown).

The response of the toxin-treated portal vein to 100 μM-GTP-γ-S (Fig. 5*B*) was similar to that seen in anococcygeus muscle. Again GTP-γ-S caused a transient contracture, albeit smaller than the noradrenaline response. Thereafter, the preparation no longer responded to noradrenaline, as with rat anococcygeus, this effect was not reversible.

The effects of GDP-β-S on the noradrenaline response

Figure 6*A* and *B* illustrates the effect of 100 μM-GDP-β-S. This non-metabolizable analogue of GDP binds to the G-protein and inactivates the protein (Williamson, 1986). As shown in Fig. 6*A*, 100 μM-GDP-β-S caused a slowing of the time course of the response to noradrenaline in toxin-treated rat anococcygeus muscle. This response, like that of GTP-γ-S was not reversible. In guinea-pig portal vein preparations 100 μM-GDP-β-S caused a reversible reduction in the size of the noradrenaline-induced tension transient, an example record is shown in Fig. 6*B*.

The effect of GTP and noradrenaline on Ca^{2+}-activated force

The responses of the two types of permeabilized smooth muscle to noradrenaline and GTP have been interpreted as a result of a release of Ca^{2+} from the SR. However, as mentioned in the introduction, noradrenaline will also stimulate phosphorylation

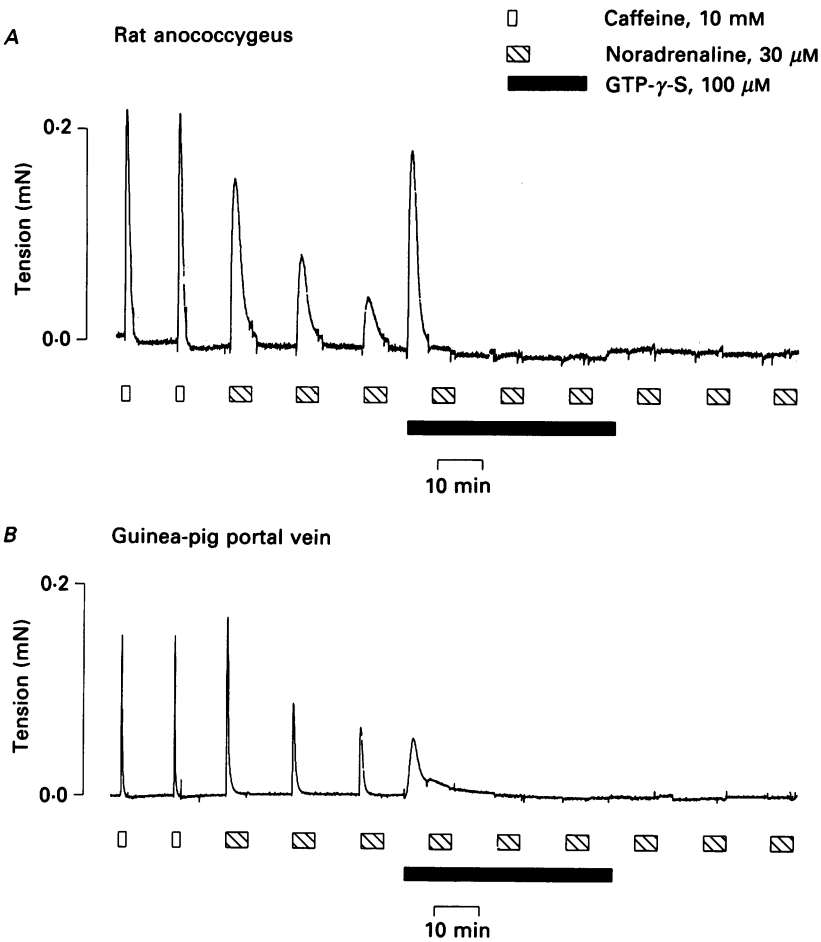

Fig. 5. The effect of 100 μM-GTP-γ-S on the noradrenaline response in permeabilized rat anococcygeus (*A*) and guinea-pig portal vein (*B*). The addition of caffeine, noradrenaline and GTP-γ-S are indicated by the bars below the respective traces.

of intracellular proteins. This may contribute to the contractile response observed by increasing the Ca^{2+} sensitivity of the contractile proteins. To assess the Ca^{2+}-sensitizing effects of noradrenaline and GTP, experiments were carried out on permeabilized smooth muscle preparations in the presence of a high concentration of the Ca^{2+} buffer EGTA (10 mM). Under these conditions caffeine is unable to elicit a response as the Ca^{2+} released from the SR is buffered by EGTA. Thus the effects of noradrenaline and GTP can be interpreted in terms of changing the relationship between $[Ca^{2+}]$ and tension.

Figure 7*A* illustrates typical responses from a permeabilized strip of rat anococcygeus muscle to progressively raising [Ca^{2+}] in the bathing solution in the presence of 10 mM-EGTA. Results of three experiments from both rat anococcygeus muscle and guinea-pig portal vein longitudinal muscle are plotted in Fig. 7*B*. Both

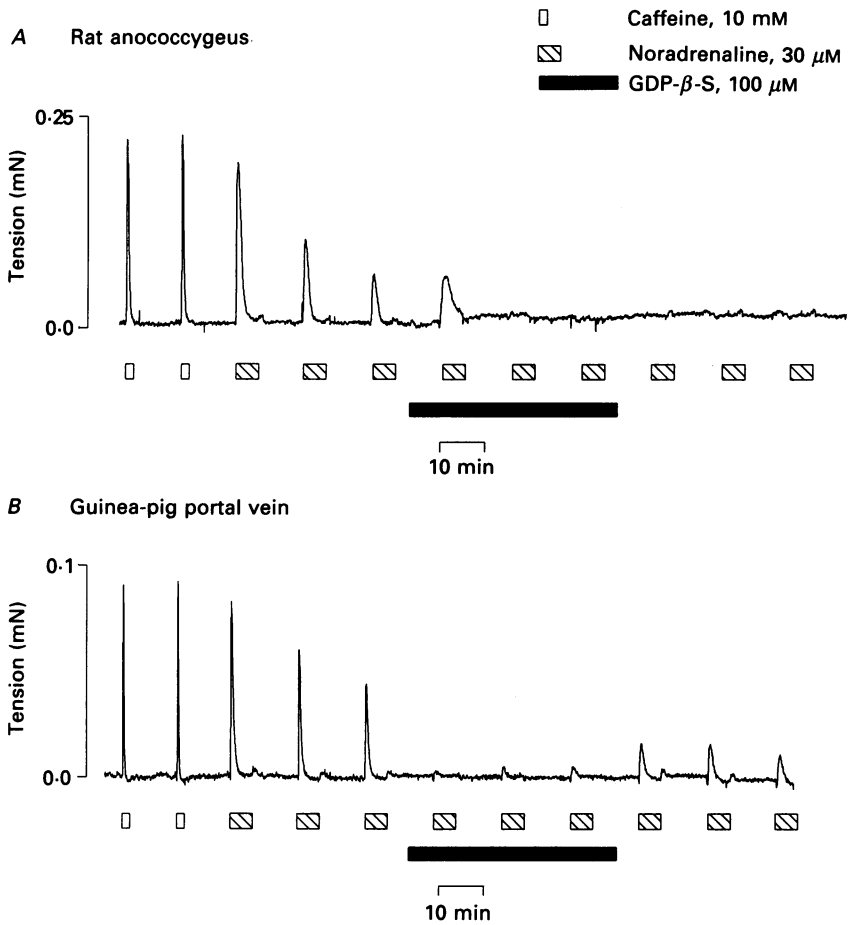

Fig. 6. The effect of 100 μM-GDP-β-S on the noradrenaline response in permeabilized rat anococcygeus (*A*) and guinea-pig portal vein (*B*). The addition of caffeine, noradrenaline and GDP-β-S are indicated by the bars below the respective traces.

tissues show similar Ca^{2+} sensitivities, with anococcygeus muscle having a slightly higher sensitivity to Ca^{2+}. The mean maximal Ca^{2+}-activated force produced by muscle strips (approximately 100 μm diameter) from rat anococcygeus muscle was 1·16 mN (±0·35 mN, $n = 5$), compared with only 0·25 mN (±0·14 mN, $n = 8$) in guinea-pig portal vein.

The effects of noradrenaline and GTP are illustrated in Fig. 8*A* and *B*. The initial tensions were achieved by equilibrating the permeabilized preparations in solutions with the [Ca^{2+}] indicated at the side of the record. The lowest [Ca^{2+}] (0·08 μM) was close to the concentration present in the experiments using 0·2 mM-EGTA. A [Ca^{2+}]

of 0·3 μM was chosen in order to generate approximately one-third maximal Ca^{2+}-activated force. The highest concentration of 100 μM-Ca^{2+} was chosen to represent the maximal Ca^{2+}-activated force that can be achieved by both preparations.

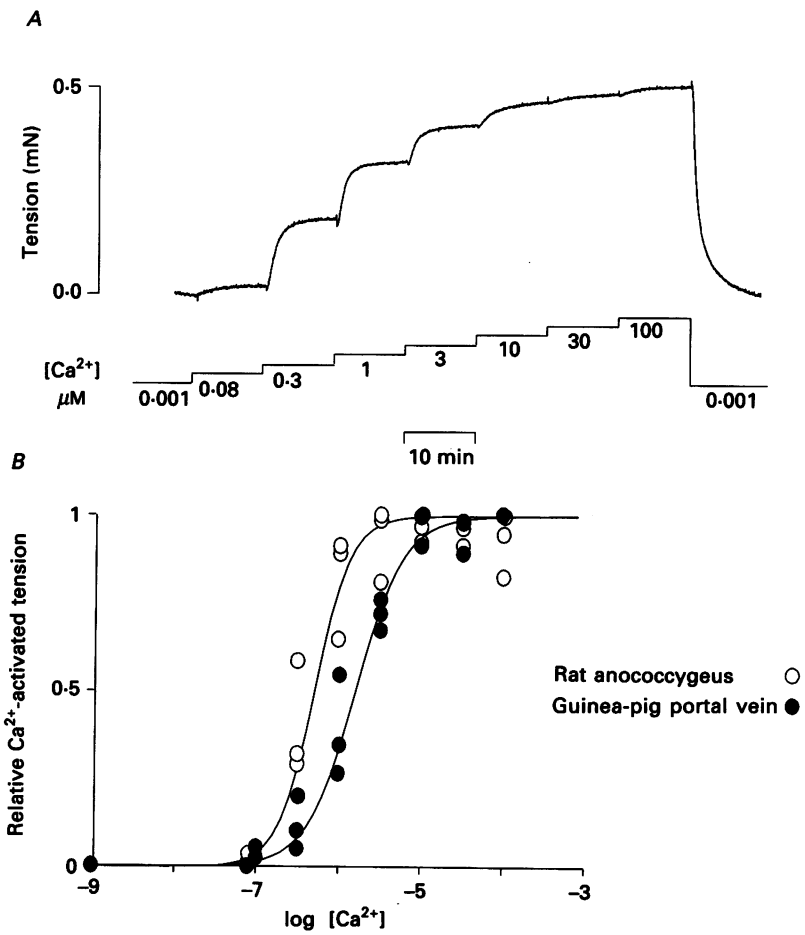

Fig. 7. The sensitivity of toxin-permeabilized smooth muscle to $[Ca^{2+}]$ in the presence of 10 mM-EGTA. Panel *A*, tension record from rat anococcygeus muscle. The $[Ca^{2+}]$ was raised progressively as indicated below the trace. Panel *B*, graph of $\log_{10}[Ca^{2+}]$ normalized tension for both rat anococcygeus and guinea-pig portal vein preparations. The data plotted are the steady-state tensions from three experiments carried out on each preparation. The continuous line represents the best-fit curve of the following form of the Hill equation:

$$T/T_{max} = (K_m \times [Ca^{2+}])^n/[1 + (K_m \times [Ca^{2+}])^n],$$

where T/T_{max} is a fraction of maximal Ca^{2+}-activated force (T_{max}); K_m is the apparent affinity constant of the myofilaments for Ca^{2+}. For data from guinea-pig portal vein data, $n = 2{\cdot}0$ and $K_m = 10^{6{\cdot}3}$. For rat anococcygeus data $n = 1{\cdot}5$ and $K_m = 10^{5{\cdot}8}$.

Noradrenaline and GTP were applied in a similar protocol to that used in Fig. 2*A* and *C*. The tension records at each $[Ca^{2+}]$ were obtained from separate preparations, for measurements at submaximal Ca^{2+}-activated forces, the maximum force was initially recorded before equilibration at the lower $[Ca^{2+}]$.

In rat anococcygeus, noradrenaline caused a transient increase in Ca^{2+}-activated force in the presence of 0·3 and 100 μM-Ca^{2+}; only a very small effect was observed in 0·08 μM-Ca^{2+}, this would represent less than 5% of the mean amplitude of the responses seen in 0·2 mM-EGTA. The addition of 100 μM-GTP caused an increase in

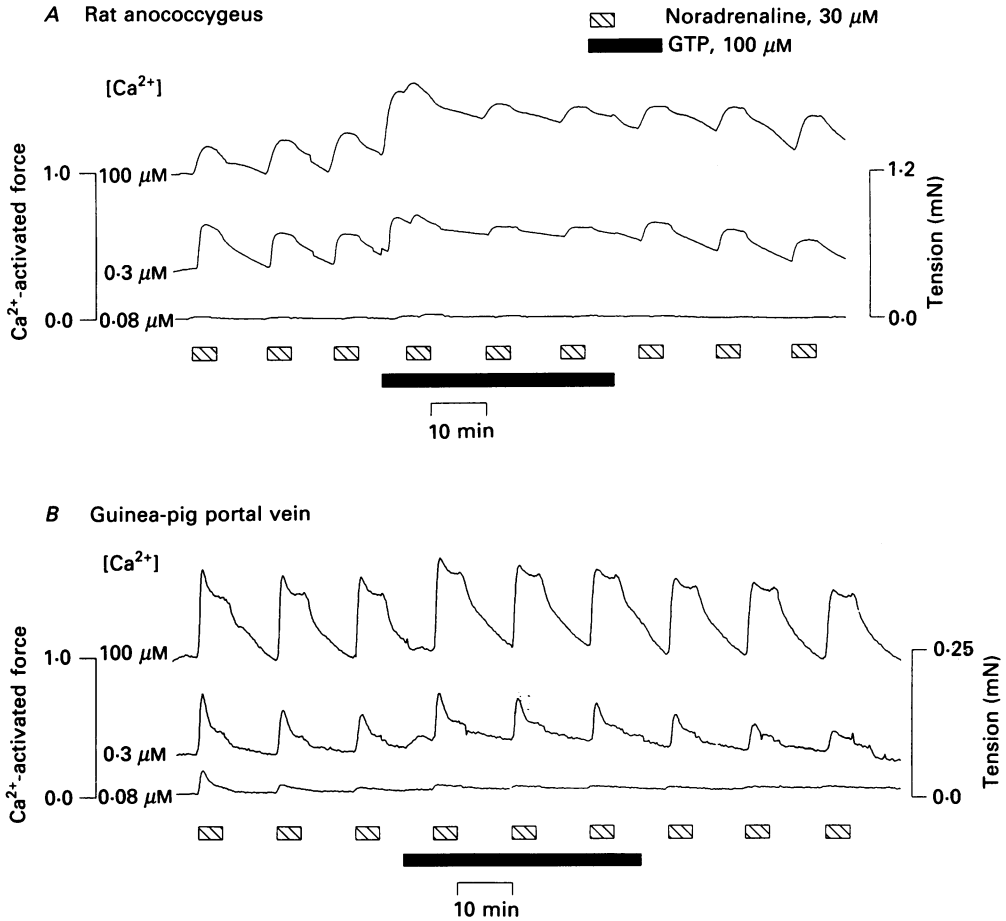

Fig. 8. The effect of GTP and noradrenaline on Ca^{2+}-activated force in toxin-permeabilized smooth muscle preparations. Addition of GTP and noradrenaline is indicated by the bars below each trace. The [Ca^{2+}] present before the addition of noradrenaline is indicated at the side of each record. Panel *A*, results from three rat anococcygeus preparations. Panel *B*, results from three guinea-pig portal vein experiments.

Ca^{2+}-activated force; application of noradrenaline in the presence of GTP caused only a small additional enhancement of force. This effect appeared to be partially reversible, since the size of the noradrenaline response increased on removal of GTP. Figure 8*B* shows typical results from guinea-pig portal vein. Small transient responses are seen on addition of noradrenaline in the presence of 0·08 μM-Ca^{2+}. The largest of these would represent approximately only about 15% of the mean

amplitude of the noradrenaline contracture observed in 0·2 mM-EGTA. In the presence of 0·3 μM-Ca^{2+}, the addition of noradrenaline caused an initial transient contracture which decayed to a steady level higher than the control level. The addition of GTP caused only a small contracture relative to the response seen in rat anococcygeus preparations. The form of the noradrenaline response in the presence of GTP was little different from that seen in the absence of GTP. Unlike the results from rat anococcygeus preparations, noradrenaline caused a much larger enhancement of Ca^{2+}-activated force in 100 μM-Ca^{2+} than in 0·3 μM-Ca^{2+}. The results shown in Fig. 8 were typical of a further six experiments carried out on both preparations. These results are not plotted on a curve similar to that shown in Fig. 7*B* for two reasons: (i) the response to both noradrenaline and GTP was complex, with frequently no steady-state value, (ii) repeated application of noradrenaline gave progressively smaller responses (Fig. 8), and so a number of results could not be reliably obtained from one preparation.

DISCUSSION

This study is the first to examine the response of toxin-treated smooth muscle to repetitive applications of noradrenaline. The results described above show some important differences from those previously published on toxin-permeabilized smooth muscle.

The effect of GTP on the noradrenaline response

GTP (100 μM) caused a large transient contracture in toxin-treated anococcygeus muscle. If GTP was applied after repeated noradrenaline responses had shown significant decline, it enhanced the size of the next noradrenaline response. But if the GTP was applied after the first noradrenaline response, the second noradrenaline response was not larger, but was still significantly larger than the second response seen in the absence of GTP.

These results contrast those seen in strips of toxin-treated portal vein. In this tissue GTP did not cause a significant contracture, yet in its presence the noradrenaline response was markedly enhanced. This occurred even when GTP was added after only one exposure to noradrenaline. This latter result is similar to that seen by Kobayashi *et al.* (1988).

Possible reasons for the decline in the noradrenaline response

The response to noradrenaline declined after successive exposures to noradrenaline. In rat anococcygeus preparations the decline in the size of the noradrenaline response was irreversible. This decline was not accompanied by a fall in the size of the caffeine-induced contracture. The simplest interpretation of this observation is that the fall in the noradrenaline response was not accompanied by a fall in the Ca^{2+} content of the SR. However, as mentioned in the Methods section, caffeine can have additional effects through raising the concentration of cyclic AMP within the preparation. Thus an alternative interpretation of Fig. 3 may be possible involving the modulation of the effects of cyclic AMP by either the application of noradrenaline or GTP. However, the results shown in Fig. 4 support the simpler explanation of the effects

of caffeine. In this situation, neither the repeated application of caffeine, nor the presence of GTP appeared to alter the Ca^{2+} released from the SR by IP_3 in rat anococcygeus preparations.

The decline in the size of the noradrenaline response occurs to a much lesser extent in toxin-treated portal vein preparations than in rat anococcygeus preparations, and only in the portal vein does GTP appear to reverse the decline. The processes that are responsible for the decline in the response to noradrenaline are not known. Three possible reasons are discussed below.

Depletion of membrane stores of phosphatidylinositol 4,5-bisphosphate (PIP_2)

One possible explanation may be that membrane stores of PIP_2 may be depleted. In intact cells, the IP_3 synthesized on α_1-adrenergic stimulation has been shown to result in a temporary depletion of the membrane stores of PIP_2 (Hashimoto, Hirata, Itoh, Kanmura & Kuriyama, 1986). After α_1-adrenergic stimulation, the IP_3 is converted back to PIP_2. But in permeabilized preparations some of the IP_3 may be lost to the bathing solution. Thus repeated noradrenaline applications would gradually deplete the membrane stores of PIP_2 causing a reduction in the IP_3 produced on application of noradrenaline. While this explanation may account for some of the fall in the noradrenaline response seen in rat anococcygeus preparations, it cannot be a major factor in guinea-pig portal vein preparations since, after showing a profound fall in amplitude, responses to noradrenaline are enhanced and maintained by GTP.

Loss of intrinsic GTP

The decline of the response to noradrenaline may result from a gradual loss of intrinsic GTP from the permeabilized preparation. The results from guinea-pig portal vein are consistent with this explanation since the initial rate of fall in amplitude of the noradrenaline response is similar to that seen on removal of GTP. Also, the amplitude of the noradrenaline response is enhanced by approximately 1·5 times the size of the first noradrenaline response independent of when the GTP was added. A feature seen in all experiments with guinea-pig portal vein is that the noradrenaline response does not decay immediately after the GTP is withdrawn. Instead a smaller response is seen only after the second noradrenaline application without GTP. The cause of this behaviour is unknown and is being investigated. However, this explanation cannot account for the decay of noradrenaline response in rat anococcygeus muscle, since the decay persists in the presence of GTP.

Phosphorylation of intracellular sites

Stimulation of the G-protein may have inhibitory effects in addition to the effects on the SR. DAG released on PIP_2 break-down causes the activation of protein kinase C. This in turn leads to the phosphorylation of a number of intracellular proteins including G-protein (Katada, Gilman, Watanabe, Bauer & Jakobs, 1985; reviewed in Sagi-Eisenberg, 1989) and even the adrenoceptor (Benovic, Strasser, Caron & Lefkowitz, 1986; Sagi-Eisenberg, 1989). Phosphorylation of the G-protein would lessen its ability to increase phospholipase C activity. Subsequent applications of noradrenaline would then have smaller effects since a greater fraction of the

population of G-proteins would be phosphorylated. In intact cells the intracellular proteins are dephosphorylated, ensuring that the response to noradrenaline does not decline. However, toxin-treatment of rat anococcygeus preparations may disrupt these processes.

The transient contracture caused by GTP in rat anococcygeus may result from the action of a subpopulation of G-proteins that are chronically activated. The net effect of GTP on the subsequent re-addition of noradrenaline would depend on the fraction of noradrenaline-activated G-proteins that are not inhibited by phosphorylation, and to what degree GTP can stimulate these remaining G-proteins. Portal vein smooth muscle appears to differ from rat anococcygeus in that (i) the noradrenaline contractures do not show such a profound and irreversible decay, (ii) GTP does not cause a significant contracture. These differences could be explained if the noradrenaline responses in portal vein preparations were not subject to inhibition by phosphorylation and these preparations lacked chronically activated G-protein.

The effect of IP_3

Figure 5 shows IP_3 induced tension transients with similar time courses to noradrenaline responses. The sensitivity of rat anococcygeus to exogenous IP_3 was similar to that reported in smooth muscle from rabbit main pulmonary artery (Somlyo *et al.* 1988) and rabbit mesenteric artery (Saida *et al.* 1988). Responses to IP_3 were not affected by either GTP or intervening exposures to noradrenaline. The absence of an effect of GTP on the IP_3 response is contrary to the report by Saida *et al.* (1988). However, these workers carried out procedures to minimize intrinsic GTP production. As illustrated in Fig. 10, the addition of GTP would be expected to sensitize the preparation to Ca^{2+}, but this effect would be small at the tensions achieved during both caffeine and IP_3 responses in rat anococcygeus preparations. Iino (1987) described results suggesting that the Ca^{2+} released from the SR by caffeine represents only part of the total releasable Ca^{2+} in the SR: IP_3 could induce Ca^{2+} release from a fraction of the SR that was not accessed by caffeine. If similar compartments exist in the SR of rat anococcygeus preparations, the responses shown in Fig. 5*C* would indicate that while amplitude of the noradrenaline response fell, this was not accompanied by a fall in the Ca^{2+} content of the fraction of the SR accessed by IP_3 alone.

The effect of GTP-γ-S

The non-metabolizable form of GTP, GTP-γ-S, will bind to and activate G-proteins even in the absence of an agonist such as noradrenaline. This causes the release of IP_3 and thus the release of Ca^{2+} from the SR. As shown in Fig. 7*B*, GTP-γ-S causes a transient contracture in toxin-permeabilized guinea-pig portal vein. Consequently, and presumably because the G-protein is permanently activated, the preparation is no longer responsive to noradrenaline. An identical response to GTP-γ-S is seen in rat anococcygeus muscle, the time course of the contracture to GTP-γ-S is similar to that observed with GTP. This result supports the notion that 100 μM-GTP activates a number of chronically active G-proteins present in rat anococcygeus preparations: the remaining G-proteins are not activated unless noradrenaline is present, and both these subpopulations are activated by GTP-γ-S.

The effect of GDP-β-S

The reversible reduction of the noradrenaline response by 100 μM-GDP-β-S in toxin-permeabilized guinea-pig portal vein is readily explicable in terms of the ability of GDP-β-S to reduce the activity of G-proteins. The GDP-β-S binds to the G-protein and prevents the release of GDP. Under these circumstances the G-protein remains inactivated. However, it is difficult to see how this mechanism can explain the results observed in rat anococcygeus where GDP-β-S did not immediately inhibit the already small noradrenaline response. The eventual inhibition of the noradrenaline response in rat anococcygeus preparations could be simply due to the processes that cause the normal decay of the response. This may explain the lack of recovery of the response on removal of GDP-β-S.

The effect of noradrenaline and GTP on Ca^{2+}-activated force

The experiments on Ca^{2+}-activated force indicate that noradrenaline and GTP can have profound effects on Ca^{2+}-activated force in both permeabilized smooth muscle preparations. The tension response in 0·2 mM-EGTA is due to a combination of an increase in $[Ca^{2+}]$ within the preparation (as a result of Ca^{2+} release from the SR) and an increased response of the myofilaments to Ca^{2+}. The small effect of GTP on the noradrenaline response with 10 mM-EGTA in guinea-pig portal vein preparations contrasts with the results in 0·2 mM-EGTA, where GTP caused a marked increase in the noradrenaline response. This observation confirms the proposal that GTP enhances noradrenaline induced Ca^{2+} release, rather than enhancing the response of the myofilaments to a similar Ca^{2+} release. The relationship between $[Ca^{2+}]$ and tension shown in Fig. 7*B* for portal vein preparations is similar to that reported by Kitazawa *et al.* (1989) in the same preparation, and in rabbit mesenteric artery by Fujiwara, Itoh, Kubota & Kuryyama (1989) (saponin-permeabilized) and Nishimura *et al.* (1988) (toxin-permeabilized). All three studies showed that GTP (100 μM) enhanced the Ca^{2+} sensitivity. However, only Kitazawa *et al.* (1989) indicated that GTP enhanced maximal Ca^{2+}-activated force. In the present study 100 μM-GTP increased maximal force by 5·0 and 5·8% in two experiments on guinea-pig portal vein and by 29·3 and 21% in anococcygeus. Similar effects of noradrenaline on Ca^{2+}-activated force were observed by Nishimura *et al.* (1988) on toxin-permeabilized rabbit mesenteric artery, and Kitazawa *et al.* (1989) on toxin-permeabilized guinea-pig portal vein.

Again, the results from rat anococcygeus preparations were different to those seen in guinea-pig portal vein preparations and to those effects reported in the literature for other smooth muscle preparations. GTP enhanced force, but the response to noradrenaline was reduced in the presence of GTP.

In conclusion, this study shows that toxin-permeabilized smooth muscle preparations from the rat anococcygeus and guinea-pig portal vein do not respond in similar ways to either noradrenaline or GTP. The responses seen in guinea-pig portal vein are consistent with noradrenaline acting on α_1-receptors and initiating the synthesis and release of IP_3 and DAG via a G-protein-mediated mechanism. A similar system appears to be present in rat anococcygeus, with a subpopulation of G-proteins that appear to be activated in the absence of adrenergic agonists. However,

the response to noradrenaline is progressively inhibited via a mechanism that is poorly understood, and further investigations are required to clarify this effect.

We would like to thank Francis Burton and, Drs Susan Wray, David Eisner and Vincent Wilson for their criticisms of earlier forms of this manuscript and Dr David Miller for laboratory facilities. In addition, we thank Dr David Miller, Professor Miles Housley, Dr Graham Milligan and Professor John McGrath for their valuable discussions. This work was supported financially by the British Heart Foundation. C.A.C. is a Glasgow University Medical Faculty Scholar.

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