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Intracellular Ca^{2+} and contractile responses to α_1-adrenoceptor subtype activation in rat aortic vascular smooth muscle

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Abstract

To simultaneously and rapidly measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and contraction in vascular smooth muscle, the Ca^{2+} fluorophore, fura-2/acetoxymethyl ester, was incorporated into an intact sample of rat aorta. Noradrenaline produced a biphasic $[Ca^{2+}]_i$ response (phase-1 and phase-2) which was different to the monophasic contractile response. Phase-1 of the $[Ca^{2+}]_i$ response was a large, fast, transient increase which usually clearly preceded contraction. Phase-2 of the $[Ca^{2+}]_i$ response was slower, peaked between 20–40 s after addition of noradrenaline, and often subsequently declined whilst contraction continued to increase. Contraction followed phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline more closely than phase-1. WB 4101 (α_{1A}-adrenoceptor antagonist) produced a major reduction in phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline, a lesser reduction of phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline and least reduction of contraction. Chlorethylclonidine (α_{1B}-adrenoceptor antagonist) reduced phase-1 and phase-2 of the $[Ca^{2+}]_i$ response and contraction to noradrenaline to a similar degree. We conclude that noradrenaline produces a biphasic $[Ca^{2+}]_i$ increase and that neither α_1-adrenoceptor subtype is specifically linked to phase-1 or phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline in the rat aorta. However, selective α_{1B}-adrenoceptor activation shows a higher force/$[Ca^{2+}]_i$ relationship in comparison to α_{1A}-adrenoceptor activation.

Keywords: α_1-Adrenoceptor, subtype; Ca^{2+} concentration, intracellular; Contraction; Smooth muscle, vascular; Fura-2

1. Introduction

An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has long been considered to cause contraction in vascular smooth muscle although the precise relationship between these two parameters is unclear. The first simultaneous recordings of $[Ca^{2+}]_i$ and contraction in vascular smooth muscle were achieved using aequorin loaded into individual cells of intact tissue either by microinjection or by reversible permeabilization. These studies demonstrated that the force/light ratio was greater for noradrenaline than for KCl and it was suggested that this was due to the noradrenaline induced Ca^{2+} response being more effective than that for KCl at producing muscle contraction (Morgan and Morgan, 1982, 1984).

The study of $[Ca^{2+}]_i$ has been greatly assisted by the development of Ca^{2+} fluorophores, in particular fura-2, developed by Tsien and his colleagues (Grynkiewicz et al., 1985) which, when combined with acetoxymethyl ester groups, can be incorporated directly into cells (Tsien, 1981; Tsien et al., 1985). In 1988, three separate research groups confirmed that an increase in $[Ca^{2+}]_i$ coincided with contraction in smooth muscle (Bruschi et al., 1988; Himpens and Somlyo, 1988; Karaki et al., 1988; Sato et al., 1988a,b). Subsequent studies have also confirmed that noradrenaline is more efficient at producing force from a given increase in $[Ca^{2+}]_i$ in comparison to KCl (Jensen et al., 1992; Vonderlage and Schreiner, 1989; Yoshitake et al., 1991).

The rat aorta contains primarily α_1-adrenoceptors, which are thought to mediate contraction via intracellular Ca^{2+} release and extracellular Ca^{2+} influx. However, the receptor has characteristics which suggested the existence of two subclasses of the receptor, the α_{1A} and the α_{1B} subtype (McGrath, 1982). This was later confirmed with radioligand binding studies using WB 4101 (Morrow and Creese, 1986) and chlorethylclonidine (Johnson and Minneman, 1987) in the rat brain to identify two sub-populations of α_1-adrenoceptor subtypes. WB 4101 was designated as selectively blocking α_{1A}-adrenoceptor subtypes and chlorethylclonidine selectively blocking α_{1B}-adrenoc-

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eptor subtypes (Minneman, 1988). Much work has examined these subtypes and has shown that the proportion and the properties of the subtypes appear to vary amongst different tissues (Hieble et al., 1986; Kong et al., 1994; Minneman et al., 1983; Piascik et al., 1993). WB 4101 and chlorethylclonidine have thus far been considered the antagonists of choice for selective inhibition of α_{1A}- and the α_{1B}-adrenoceptor subtype respectively (Jiang et al., 1995; Suzuki et al., 1990a,b; Terman et al., 1990). Although there are other antagonists which have higher selectivity for either subtype, they also have intrinsic activity which make them unsuitable for functional studies in intact tissue.

Activation of the α_{1A}-adrenoceptor subtype has been associated with extracellular Ca^{2+} influx through 1,4-dihydropyridine-sensitive Ca^{2+} channels, while α_{1B} subtype activation has been associated with intracellular Ca^{2+} release (Han et al., 1987; Minneman, 1988; Suzuki et al., 1990b). This conclusion was supported by contractile studies showing that WB 4101 sensitive α_1-adrenoceptors were dependent upon extracellular Ca^{2+} influx, whereas chlorethylclonidine sensitive α_1-adrenoceptors were not (Han et al., 1990a; Hanft and Gross, 1989). However, studies utilising [^{3}H]inositol phosphate have clearly demonstrated that either the α_{1A}- or the α_{1B}-adrenoceptor subtype, or both, is able to induce inositol phosphate formation (Esbenshade et al., 1993; Han et al., 1990b; Lazou et al., 1994; Michel et al., 1993; Piascik et al., 1990; Wilson and Minneman, 1990). Functional and receptor protection studies have suggested that only one α_1-adrenoceptor subtype is responsible for both extracellular Ca^{2+} influx and intracellular Ca^{2+} release (Oriowo et al., 1992; Oriowo and Ruffolo, 1992; Piascik et al., 1990). Overall, it can perhaps be concluded that α_{1A}-adrenoceptors may be preferentially linked to extracellular Ca^{2+} entry, and α_{1B}-adrenoceptors to intracellular Ca^{2+} release in some circumstances, but this is clearly by no means a universal phenomenon.

The aim of the work presented here was to develop a system which could rapidly and simultaneously measure $[Ca^{2+}]_i$ and contraction in vascular smooth muscle, thus enabling the $[Ca^{2+}]_i$/time relationship and the contraction/time relationship when the muscle was stimulated with noradrenaline to be studied in more detail than previously reported. We then aimed to investigate whether activation of either the α_{1A}- or α_{1B}-adrenoceptor subtype produced different patterns of $[Ca^{2+}]_i$ changes and different force/$[Ca^{2+}]_i$ relationships in the rat aorta, and in particularly whether one subtype sensitised the contractile apparatus to the increase in $[Ca^{2+}]_i$.

2. Materials and methods

Sprague-Dawley rats (220–320 g) of either sex were used in all experiments. The rats were killed in a CO_2 chamber and the descending aorta was removed, flushed, and placed in normal physiological saline solution (PSS) of the following composition (mM): Na^+, 137.4; K^+, 5.4; Mg^{2+}, 1.2; SO_4^-, 1.2; HCO_3^-, 15.0; $H_2PO_4^-$, 1.2; Cl^-, 129.1; glucose, 11.5; Ca^{2+}, 2.5. All solutions were bubbled with carbogen (95% O_2/5% CO_2) which maintained a pH of 7.4. The adventitia was removed using dissecting scissors and a scalpel. In order to ensure removal of the endothelium, the lumenal surface was scraped with a triangular needle. Histological examination of the tissues demonstrated that the muscle wall was 7–10 cell rows thick, the endothelium had been successfully removed, and there was only a small amount of remaining adventitia.

An organ bath arrangement was designed to allow the simultaneous measurement of $[Ca^{2+}]_i$ and muscle contraction within the confines of a cuvette and spectrophotometer. Muscle tension was measured by attachment to a force transducer (Grass FT03C) and a polygraph (Grass Mode 7-D). Open ring preparations were used, and held in place and flat by two modified clamps, the lower one fixed and the upper one attached to the force transducer. The final arrangement of the open ring preparation in the cuvette/organ bath is shown in Fig. 1. The organ bath was a $1 \times 1 \times 4$ cm quartz cuvette with the force transducer fixed directly above the cuvette chamber and adjusted by a screw thread to induce changes in muscle tension without significant movement in the muscle sample. The temperature of the cuvette chamber was regulated by attachment to an external water bath.

After equilibration for 45 min at 37°C during which the tissue relaxed to a base tension of 1 g, successive challenges with 80 mM KCl were then performed in order to assess contractile strength. An autofluorescent test was performed to determine (a) the level of background fluorescence and (b) the extent of movement artefact (frequently there was a slight parallel shift in the 340 nm and 380 nm signals).

The loading solution consisted of the PSS which also contained 15 μM fura-2, 0.5% Cremophore-EL and 0.5%

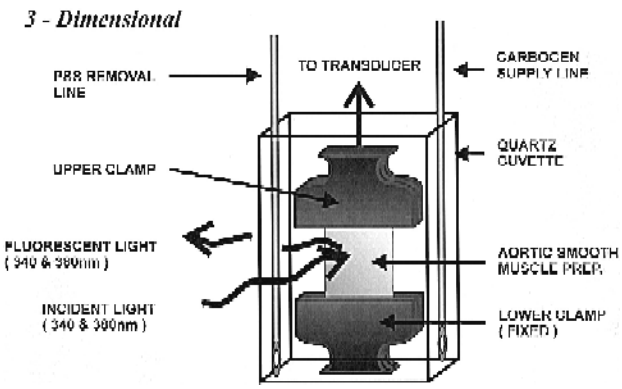

Fig. 1. Diagrammatic representation of the complete apparatus and tissue arrangement within the cuvette/organ bath. The lower clamp is attached to the wire frame and therefore is fixed and the upper clamp is attached to the force transducer. PSS is physiological saline solution.

dimethyl sulfoxide (DMSO). The muscle was loaded in the fura-2/acetoxymethyl ester containing PSS for 3 h and the incubation commenced at 15–17°C and was allowed to warm naturally to a temperature of approximately 26–27°C in order to facilitate fura-2 loading (Roe et al., 1990).

Fura-2 gave consistent $[Ca^{2+}]_i$ signals for at least 1 h after the muscle was returned to normal PSS following each incubation period (Mitsui et al., 1993). Reloading was undertaken in the same manner as the initial loading except that, prior to reloading, the solution was oxygenated and cooled, as was the muscle, to room temperature. The reloading period was only 1–2 h, depending on the preceding decrease in the intensity of the excitation signals.

The spectrophotometer used was a Fluorolog-2 with a DM1B 2000 computer manufactured by Spex Industries, USA. The instrument also included a xenon 450 W lamp, two monochromators (S1 and S2) for dual wavelength recording, a reference detector (R1 and R2) to correct for variations between S1 and S2 and a rotating chopper which allowed rapid alternation between S1/R1 and S2/R2. The spectrophotometer measured fluorescence at a 'front face' (21°) orientation.

S1 was set at 340 nm which is the wavelength fura-2 is excited by when it is complexed to Ca^{2+} and S2 was set at 380 nm which is the wavelength fura-2 is excited by in its free, or un-complexed state. Both bound and free fura-2 emit light at 505 nm (Grynkiewicz et al., 1985; Tsien, 1989). The fura-2 340/380 nm ratio (i.e. fura-2–Ca^{2+}/fura-2) was used as the measure of $[Ca^{2+}]_i$ because it is not affected by variability in tissue thickness, dye concentration or movement artefact (Grynkiewicz et al., 1985; Roe et al., 1990; Tsien, 1989; Tsien et al., 1985). In addition the conversion equations (Grynkiewicz et al., 1985) may not be valid in intact tissue preparations (Cobbold and Rink, 1987; Sato et al., 1988a).

The single concentration challenges of noradrenaline examined were 10^{-6} M noradrenaline. Although this concentration did not usually produce the maximum contractile response, higher concentrations increased the possibility of tachyphylaxis and also increased the time required for the contraction to relax and return to baseline. In any one given loading period, at least three noradrenaline single concentration challenges could be performed within the 1 h time constraint imposed by the fura-2 fluorescence decline.

The determination of the $[Ca^{2+}]_i$ responses to noradrenaline (phase-1 or phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline) was undertaken by an area under the curve (AUC) analysis. This methodology calculates the AUC from the concentration-response curve between arbitrarily defined limits by application of the trapezoidal rule (Bury, 1984). Phase-1 of the $[Ca^{2+}]_i$ response usually had peaked within 5–6 s of the instigation of the $[Ca^{2+}]_i$ increase whilst phase-2 of the $[Ca^{2+}]_i$ response usually peaked between 20–40 s after the initiation and then slowly declined. Therefore the arbitrarily defined limits for phase-1 were the AUC from time 0–10 s, using the time points 0, 5 and 10 s, and for phase-2 of the $[Ca^{2+}]_i$ response were the AUC from 10–90 s, using the time points 10, 50 and 90 s.

To determine the effects of the antagonists (WB 4101 or chlorethylclonidine), the decreased contraction and Ca^{2+} responses were firstly calculated as a percentage of their respective control responses (see below). The results were then combined to calculate the mean and S.E.M.

2.1. Assessment of effects of the antagonists

The concentration of WB 4101 required to produce a significant reduction of the response to 10^{-6} M noradrenaline was found to be 3×10^{-9} M. The antagonism of WB 4101 was reversible and an incubation time of 15 min was sufficient to obtain the full effect. Each experiment with WB 4101 was performed in the following order: a control response to 10^{-6} M noradrenaline followed by the response to 10^{-6} M noradrenaline performed in the presence of 3×10^{-9} M WB 4101 (15 min incubation).

Chlorethylclonidine antagonism was found to be time dependent and a 30-min incubation with 10^{-6} M chlorethylclonidine was found to be necessary for a consistent degree of contractile inhibition. Therefore the experimental protocol was as follows: Two responses to 10^{-6} M noradrenaline were performed 30 min apart (1st experimental set; control 1 and control 2) after which the muscle was reloaded with fura-2. After this, a third control response to 10^{-6} M noradrenaline was performed (control 3) which was followed by a final response to 10^{-6} M noradrenaline performed after a 30-min incubation with 10^{-6} M chlorethylclonidine (2nd experimental set). The noradrenaline responses in the presence of chlorethylclonidine were calculated as a percentage of the control noradrenaline (control 3) which had been corrected for any change that occurred between the control 1 and control 2 noradrenaline responses (1st experimental set).

2.2. Drugs

Noradrenaline (Sigma) was dissolved in distilled water with the addition of ascorbic acid (0.1%) to reduce the progression of oxidation. WB 4101 and chlorethylclonidine (Calbiochem) were dissolved to form a stock solution using distilled water. Although the drugs are not known to degrade quickly, nor to be light sensitive, the stock solutions were kept in light resistant containers at 4°C.

3. Results

An example of the changes in $[Ca^{2+}]_i$ and contraction produced by a single concentration of noradrenaline is given in Fig. 2A. It can be seen that a single challenge of noradrenaline produced a biphasic $[Ca^{2+}]_i$ response that was clearly different from the associated contraction which was rarely clearly biphasic. Fig. 2B shows the same responses as in Fig. 2A but with an expanded time scale.

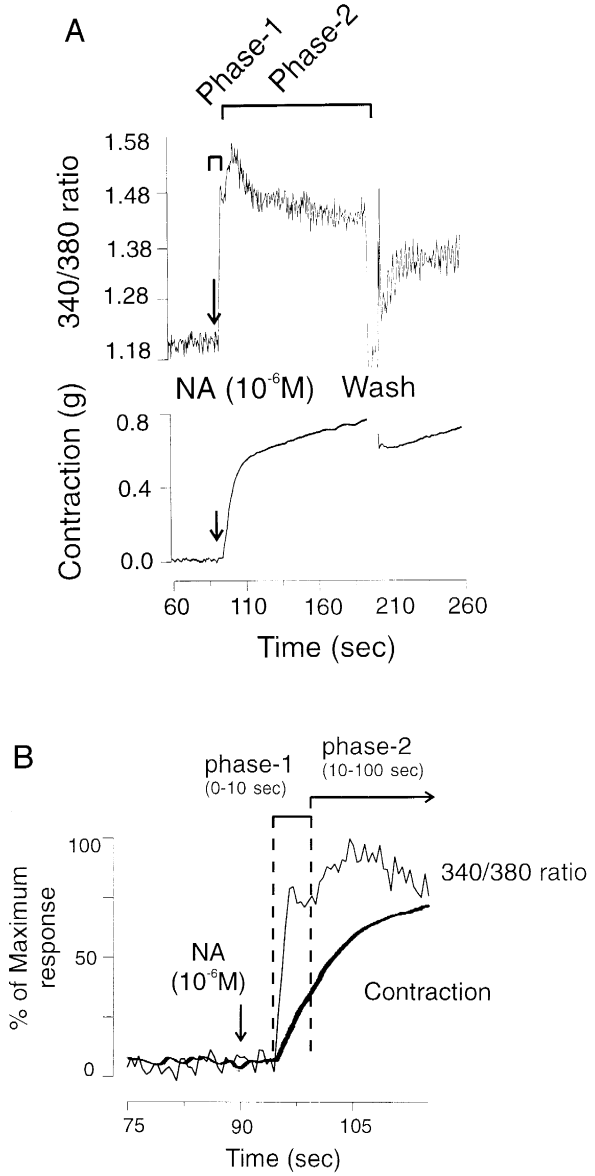

Fig. 2. (A) Typical example of the effects of the time-course of the addition of 10^{-6} M noradrenaline (NA) on contraction (lower tracing, measured in g) and on the fluorescent 340/380 nm ratio (upper tracing, measured in arbitrary units) in the rat aorta. The phase-1 and phase-2 $[Ca^{2+}]_i$ were taken as indicated. The points of addition and washing are shown. (B) Adaptation of A, showing the noradrenaline responses over an expanded time scale. Both contraction and $[Ca^{2+}]_i$ (340/380 nm ratio) responses are presented as a percentage of their maximum response.

It can be clearly seen that there were clear dissociations between the $[Ca^{2+}]_i$/time relationship and the contraction/time relationship. Firstly the commencement of phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline usually preceded, and at the latest coincided with, the start of contraction and peaked well before the force development was well established (Fig. 2B). In the example shown, at the end of phase-1 of the $[Ca^{2+}]_i$ increase, which was of near maximum intensity, the tension produced was only approximately 20–30% of the maximum contractile response. Secondly phase-2 of the $[Ca^{2+}]_i$ response, after reaching a peak, often decreased in intensity whilst contraction continued to increase (as in this example).

Fig. 3 shows the results for noradrenaline plus 3×10^{-9} M WB 4101 ($n = 8$) and noradrenaline plus 10^{-6} M chlorethylclonidine ($n = 5$). The results show phase-1 and phase-2 of the $[Ca^{2+}]_i$ responses and the contraction as a percentage of their respective responses during the control noradrenaline challenge.

It can be seen that in the presence of 3×10^{-9} M WB 4101, the AUC for phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline was reduced to $52.4 \pm 3.4\%$, and the AUC for phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline was decreased to $68.1 \pm 9.4\%$ ($n = 8$), of the control response. Both reductions were statistically significant. The maximum contractile response was not reduced significantly in the presence of 3×10^{-9} M WB 4101 ($92.8 \pm 9.5\%$, $n = 8$) and the reduction in the AUC for phase-1 of the $[Ca^{2+}]_i$ response was significantly different from the reduction in maximum contraction ($P < 0.005$). However, the difference between the reduction of contraction and the AUC for phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline did not reach statistical significance ($P = 0.09$).

In the presence of 10^{-6} M chlorethylclonidine the AUC for phase-1 of the $[Ca^{2+}]_i$ response was substantially reduced to $41.1 \pm 10.4\%$ of the control and phase-2 of the $[Ca^{2+}]_i$ response was reduced to $61.5 \pm 11.6\%$ of the control ($P = 0.005$ and 0.03, respectively, $n = 5$). The maximum contractile response and both phases of the $[Ca^{2+}]_i$ response to noradrenaline were reduced to a similar degree in the presence of chlorethylclonidine. This indicated that the force/$[Ca^{2+}]_i$ relationship was not affected by chlorethylclonidine.

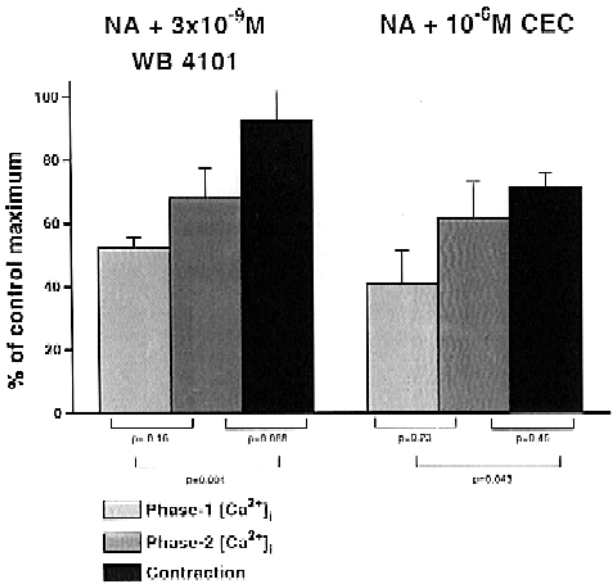

Fig. 3. Combined results of phase-1 and phase-2 of the $[Ca^{2+}]_i$ responses and the contractile response stimulated by 10^{-6} M noradrenaline (NA), in each case calculated as a percentage of the maximum response of the control, after pretreatment with 3×10^{-9} M WB 4101 ($n = 8$) or 10^{-6} M chlorethylclonidine (CEC; $n = 5$).

In addition to the above, the development of force was slower in the presence of WB 4101. The contraction produced after 10 s in the control noradrenaline challenges was $58.9 \pm 4.8\%$ ($n = 8$) of the maximum, and this was reduced to $40.0 \pm 5.4\%$ ($n = 8$) in the presence of 3×10^{-9} M WB 4101 ($P < 0.05$). WB 4101 also altered the force/$[Ca^{2+}]_i$ relationship. It is therefore clear that the maximum $[Ca^{2+}]_i$ response was reduced more than the maximum contractile response in the presence of WB 4101, and thus the force/$[Ca^{2+}]_i$ relationship was increased. The contraction/time relationship was not greatly affected in the presence of chlorethylclonidine. The contraction produced after 10 s for the control was $55.0 \pm 5.2\%$ of the maximum response and in the presence of 10^{-6} M chlorethylclonidine was $44.4 \pm 1.9\%$ of the maximum response ($P = 0.11$, $n = 5$).

4. Discussion

Noradrenaline is thought to induce contraction in vascular smooth muscle both by opening membrane Ca^{2+} channels and allowing extracellular Ca^{2+} influx and also by inducing Ca^{2+} release from intracellular stores (Cauvin and Malik, 1984; Somlyo and Himpens, 1989). In the rat aorta, there are only α_1-adrenoceptors present (Dashwood and Jacobs, 1985; Stephenson and Summers, 1987), and there is also a small population of both β_1- and β_2-adrenoceptors which can cause relaxation (O'Donnell and Wanstall, 1984). Previous studies investigating $[Ca^{2+}]_i$ and contraction simultaneously have reported that noradrenaline induces an increase in $[Ca^{2+}]_i$ which closely correlates with the contractile response (Bruschi et al., 1988; Jensen et al., 1992; Sato et al., 1988a,b) However, a biphasic $[Ca^{2+}]_i$ response stimulated by noradrenaline has not been previously described. The biphasic $[Ca^{2+}]_i$ response to noradrenaline in this study was usually not reflected in the contraction although noradrenaline has been previously described as producing a biphasic contractile response in the rat aorta and vas deferens (Heaslip and Rahwan, 1982; Major et al., 1989).

The initiation of phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline usually preceded, and at the latest coincided with, the initiation of contraction. This confirms that the rise in $[Ca^{2+}]_i$ is likely to be responsible for the contraction. However, often phase-1 of the $[Ca^{2+}]_i$ response had begun to decline before the contraction was well developed. In addition, the overall correlation between phase-2 of the $[Ca^{2+}]_i$ response and the contractile response was better than the correlation between phase-1 of the $[Ca^{2+}]_i$ response and the contractile response. This suggests that phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline, although being of considerable intensity, was not contributing significantly as a Ca^{2+} source to produce contraction and that phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline was the predominant source of contractile producing Ca^{2+}.

Comparing the results from the two selective antagonist would suggest firstly that there is a link between stimulation of the α_{1A}-adrenoceptor subtype and phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline and also the speed of contraction. Secondly, stimulation of the α_{1B}-adrenoceptor subtype as occurs with blockade of the α_{1A}-adrenoceptor subtype using WB 4101 produces a greater contractile response per increase in $[Ca^{2+}]_i$, and hence a greater force/$[Ca^{2+}]_i$ relationship, compared to stimulation of the α_{1A}-adrenoceptor subtype. Since activation of the α_{1B}-adrenoceptor subtype has been linked to Ca^{2+} release from intracellular stores such as the sarcoplasmic reticulum via the production of inositol 1,4,5-trisphosphate (Minneman, 1988; Suzuki et al., 1990a), and the same pathway has also been linked to sensitisation of the contractile apparatus via activation of protein kinase C (Collins et al., 1992; Laporte et al., 1994), it may be that these are the mechanisms by which the α_{1B}-adrenoceptor subtype activation produces a higher degree of contractile sensitivity. However, we have examined only one concentration of noradrenaline, and a complete analysis of noradrenaline concentration-effect curves in the presence and absence of the α_1-adrenoceptor subtype antagonists would need to be performed to be sure of these conclusions.

In conclusion, we have found that noradrenaline produces a biphasic $[Ca^{2+}]_i$ response which is different to the corresponding contraction, with contraction appearing to follow the phase-2 of the $[Ca^{2+}]_i$ response more closely than phase-1 of the $[Ca^{2+}]_i$ response. Neither phase-1 nor phase-2 of the $[Ca^{2+}]_i$ response to noradrenaline is specifically linked to either α_{1A}- or α_{1B}-adrenoceptor subtype activation. However, it appears that the α_{1B}-adrenoceptor subtype is more closely linked to phase-1 of the $[Ca^{2+}]_i$ response to noradrenaline which in turn is linked to the speed of contraction. In addition, selective α_{1B}-adrenoceptor activation shows a higher force/$[Ca^{2+}]_i$ relationship in comparison to α_{1A}-adrenoceptor activation.

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