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Original article

CALCIUM-SENSITIVITY OF SMOOTH MUSCLE CONTRACTION IN THE ISOLATED PERFUSED RAT TAIL ARTERY

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Desensitization and the effects of Bay K 8644 and nifedipine on the calcium-sensitivity of smooth muscle contraction were studied in the isolated perfused rat tail artery, employing the activators noradrenaline (NA) (3μM) sand potassium chloride (KC1) (100mM). Experiments were conduced in Ca^{2+} - buffered saline. Activities were added when {Ca^{2+}} free was low (1μM) and then {Ca^{2+} }free was increased stepwise to give a Ca^{2+} concentration\response curve (CRC) There was a progressive rightward shift of the CRCs with time when a series of curves was constructed. The higher the calcium concentration to which the tissue was exposed during activation, the greater was desensitization. The progressive loss in sensitivity could be attenuated by restricting the range of free calcium used for CRCs to between 1μM and 300μM Ca^{2+}. Results were similar whether activation was by NA or high KC1. When the tissues were pre-exposed to NA (3μM) ("Priming") before constructing CRCs, desensitization was produced more quickly and thus sensitivity became more "stable". However, the {Ca^{2+}} during priming and the maximum (Ca^{2+}) in a CRC determined the stable level, high {Ca^{2+}} reducing sensitivity. Priming and maximum at 300μM ca^{2+} was optimal for avoiding progressive desensitization. Bay K 8644 (0.1μM) decreased the sensitivity to Ca^{2+} but did not alter the rate of desensitization (activated by either NA or KC1). Desensitization complicated demonstration of potentiation by Bay K 8644 in the same tissue. Nifedipine (0.1μM) decreased the sensitivity of Ca^{2+} at the first CRC but thereafter CRC's were not significantly different from their controls. Only a small degree of inhibition could be seen between consecutive curves when nifedipine was given after drug-free control responses. Thus the rat tail artery exhibits higher sensitivity to Ca^{2+} on initial contact with activators. The results suggests that desensitization at some stage in excitation-contraction coupling, possibly by Ca^{2+} overload, occurs when high extracellular {Ca^{2+}} (2.5 or 5mM) is present during activation by NA. This can be prevented by avoiding high. {Ca^{2+}}, thus allowing prolonged reproducibility of high sensitivity to Ca^{2+} which, is lost.

Key words: Ca^{2+} sensitivity, desensitization, vascular smooth muscle, noradrenaline, nifedipine, Bay K 8644, rat tail artery

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INTRODUCTION

It has been observed in the past, that there was a progressive fall in responsiveness of vascular smooth muscle, such as in the rat tail artery, with time or with consecutive periods of activation by agonists, and this observation has apparently been recognized by many investigators (Medgett and Langer, 1986; Spedding, 1985; Su et al, 1984). But the lack of information on the possible causes of the initial decline between the first and the subsequent responses, has led to the neglect of the first concentration – response curve; the second or even the third being taken as the control curve for the analysis of test drugs (Medgett and Langer, 1986; Aoki and Asano, 1986).

First curves are discarded because a significant difference usually exists between the first and the subsequent curves, thereby creating problems for analysis of the data. The basis of this difference is unclear and it is reasonable to assume that the initial state is as likely to reflect the physiological properties of the tissue as does the subsequent desensitization condition: both cannot. An earlier study (McGrath et al, 1987b) showed that this desensitization can be reversed by leaving long (>2 hour) intervals between activations, and is thus not an inevitable deterioration with time.

We have now studied some of the experimental factors, which influence the initial and subsequent sensitivity to noradrenaline, and how this is influenced by the extracellular free calcium concentration. Strategies for avoiding desensitization are examined and the role of dihydropyridine-sensitive calcium channels is investigated using the calcium channel antagonist nifedipine and the calcium channel agonist Bay K 8644 (Schramm et al, 1983; Schramm et al, 1985). A preliminary communication of some of the results of this study carried out at the University of Glasgow, has been published (Ugwu *et al*, 1987).

METHODS

Preparation of the tissue for recording perfusion pressure: 1-2cm lengths of the proximal or distal segment of the rat tail artery were prepared for recording the perfusion

pressure *in vitro*. Male Wistar rats (300 to 350 gm) were killed by a blow on the head and exsanguinations. The ventral tail artery was rapidly removed and placed in aerated 'calcium-unbuffered' modified Krebs' bicarbonate solution. The vessel was cannulated at the proximal end and subsequently mounted in a 5ml jacketed organ bath. It was perfused with, and bathed in, saline of similar composition at 37^0C.

Unbuffered saline: The 'calcium-unbuffered, saline solution was made up of the following composition (in millimolar concentration):- NaCl, 119; $NaHCO^3$, 24.8; KH_2PO_4,1.2; $MgSo_4$. 7H20, 1.2; KCl, 4.7; $CaCl_2$, 2.5; glucose, 11.1; cocaine, 4 μ M; Propranolon, 1μ; and Na_2EDTA (ethylene-diaminetetra-acetic acid disodium salt), 23μM.

Buffered saline: The 'calcium-buffered' saline solution has the following composition (millimolar unless otherwise specified):- EGTA (ethylene glycol bis- {B-aminoethyl ether} N,N,N' – tetraacetic acid), 2.5 (i.e. : $0.9g^{1-1}$) NTA (nitriol-triacetic acid, i.e. N,N-bis (carboxymethyl) glycerine, free acid); 2.5; NaCl, 111.5; NaOH,7.5; $NaHCO_3$,24.8; KH_3PO^4, 1.2; $MgSO_4$. $7H_2O$, 1.2; KCl, 4.7; CaC_2 was varied from 4.69 (for calcium buffer 1) to 2.35 (for calcium buffer 6); glucose 11.1; cocaine, 4 μ M; Propanolol, 1μM;and EDTA (ethylyenediaminetetra-acetic acid disodium salt), 23 μ M. The composition of the series of buffered salines is shown in Table 1. Another saline solution used in some experiments (examining the Ca^{2+}) dependence of the contraction induced by depolarization) had high potassium chloride, low phosphate (which allowed the use of $\{Ca^{2+}\}o \geq$ 5mM without precipitation), and had identical composition to the one described above with the following exceptions:- NaCl, 24; KCl, 100; KH_2PO_4, 0.1.

Each saline was bubbled with a gas containing 95% and 5% $C0_2$, giving a partial pressure for oxygen of 615mmHg and aa pH of 7.2 to 7.4. In one series of experi6ments (see figure 6) the oxygen tension was varied by substituting N_2 for 0_2, to give 16% and 4% 0_2 as well as 95%: after such changes, 15 minute equilibration was allowed before introducing drugs or further altering the composition of the saline

Perfusion: The preparations were tested for leakage and those which were set up for perfusion. The arterial segments were mounted in the bath vertically with the cannulated proximal end of each tissue uppermost. The free distal end opened into the solution. The lumen was perfused from a gassed reservoir (37^0C) at a constant rate of 2-3ml/min with a pulsate flow pump (Watson-Marlow peristaltic cassette pump, 501U with 501M multi-channel pump head) and the perfusion pressure was recorded. This rate of flow was shown in preliminary experiments to be adequate for recording the optimal vasoconstrictor responses to noradrenaline (NA) or to high concentration of potassium chloride (KCl).

The vasoconstrictor responses were measured as an increase in the peaks of the pulsatile perfusion pressure at constant average flow, using an Elcomatic EM751 pressure transducer and Devices recorder. The perfusatee passing through the artery via the cannular mixed freely with the identical saline solution in the organ that bathed the adventitial surface.

General Experiment Protocol: A standard stabilization period of 90 to 120mins in activator-free 'Ca unbuffered' solution was allowed before any responses were obtained. The protocol involved changing the perfusing solution by briefly stopping perfusion and switching it to new solution containing the required concentration of Ca^{2+}, NA or KCl. The bathing solution in the organ bath was replaced simultaneously with the arrival of the new perfusate. Consequently both surfaces of the tissues were always exposed to an identical medium during the experiments. (In practice, the responses to noradrenaline were not larger when it was presented to both surfaces than to the initima alone, using double cannulation {data not shown} but in order to achieve equlibrium conditions the preceeding protocol was adopted). Standard exposure of the tissues to NA or KCl with either cumulative or non-cumulative protocols was for 5 mins during which time the maximum response to that concentration of the activator was obtained. In some experiments, as indicated in the text, tissues were exposed to initial 'priming' concentrations of noradrenaline in various buffers for 5 min before starting the main protocol.

In all experiments involving responses to activators, EGTA and NTA were included (2.5mM each) so that any toxic effect would be constant throughout, and unless a particular buffers is specified total $CaCl_2$ was adjusted to keep $[Ca^{2+}]$ free at 2.5mM. In all the experiments involving NA-induced contractions, cocaine (4 μ M), propranolol (1 μ M) and EDTA (23 μ M) were present.

Noradrenaline concentration/response curves: After equlibration for 15min in the appropriate saline buffer (starting at the lowest concentration of calcium and working up), concentration/responses curves to noradrenaline were constructed non-cumulatively, with 5min contact and 10min wash, starting at the lowest concentration, 30nM, and proceeding in half log unit step to 30 μ M. 15min intervals were left between curves in different calcium buffers, giving

a routine cycle time for noradrenaline concentration/responses curves of 110min.

In these experiments the tissues were initially exposed to 3 μ M noradrenaline for 5min as part of a stabilization procedure, 15min before starting the first concentration/responses curve. In retrospect, this is likely to have caused some desensitization and this will be considered in the discussion.

Calcium concentration/responses curves: Concentration/response curves to Ca^{2+} (CCRC) were constructed by changing to the lowest [Ca^{2+}] (buffer 6; see Table 1) for 15min, adding the activator (NA or KCl) then, starting 5mins later, changing stepwise to higher [Ca^{2+}] at 5mins interval starting from buffer 6 [Ca^{2+}]o =10^{-6} M] through a series of buffers referred to as buffers 5 to 1 which took the [Ca^{2+}] to 300 μ M. In some experiments, further increments in [Ca^{2+}] free of 5.32mM or at times to 10. 32mM. Details of the [Ca^{2+}] in the various buffers are outlined in Table 1.

The concentration of NA (3μM) or KCl (100mM) was kept constant while changing [Ca^{2+}]. At the end of the construction of the CRCS, the perfusion was stopped and the bathing and perfusing solutions were replaced by activator-free buffer 6 solution and allowed to re-equilibrate (to baseline response) before constructing another CCRC. Preliminary experiments showed that washing and "resting" in buffer 6 minimized desensitation, i.e. it was worse if [Ca^{2+}] was higher. Intervals of 15min were allowed between curves in the initial protocol but this was varied in other experiments as noted in the text. A total time of 45minutes was taken to complete the construction of each control curve, giving a routine cycle of 1 curve per hour.

Table 1
Ca^{2+} concentrations in the buffers used

Buffer	[Ca^{2+}] free (M)	[Ca^{2+}] total (mM)
6	1 x 10^{-6}	2.35
5	3 x 10^{-6}	2.47
4	1 x 10^{-5}	2.69
3	3 x 10^{-5}	3.12
2	3 x 10^{-4}	3.90
1	3 x 10^{-4}	4.69
'half Ca^{2+}	1.25 x 10^{-3}	[Buff 1] + 1.57
'normal' Ca^{2+}	2.50 x 10^{-3}	[Buff 1] + 2.83
'double' Ca^{2+}	5.00 x 10^{-3}	[Buff 1] +5.32
'qudruple' Ca^{2+}	10.00x10^{-3}	[Buff 1] + 10.32

Drugs and chemicals

The following substances were used:- (-)-noradrenaline bitartrate salt (sigma), Bay K 8644 (Bayer), nifedipine (Bayer), Cocaine HCl (McCarthys), DL-Propranolol HCl (I.C.I.), E.D.T.A. (B. D. H.), E.G.T.A. (ethylene glycol bis-{B-aminoethyl ether} N,N,N'-tetraacetic acid (Sigma), N.T.A. (nitrilo-triacetic acid, i.e., N,N-bis {carboxymethyl} glycine, free acid) (Sigma). Stock solutions of drugs were dissolve in distilled water, v/v and
diluted in the appropriate saline.

***Expression of data*:** All results have been expressed, or represented on graphs, as the mean ± S.E.M. Statistical analysis was performed using Student's 't' test for paired or unpaired data, as appropriate and the 0.05 level of probability was regarded as significant.

Before sensitization occurred the preparations characteristically showed a 'peaked' concentration/response curve to agonists, or to calcium concentration at a fixed concentration of agonist. Both 'sensitivity' and maxima subsequently declined, if steps were not taken to avoid desensitization and, in many cases, a 'maximum' was not obtained within the possible range of soluble calcium. This raises problems in quantifying calcium sensitivity, since there was often no maximum, within a given curve, on which to base the 50% response for calculation of a pD_2 value. Furthermore, after desensitization, the curves sometimes did not attain 50% of the maximum from the first curve. For simplicity we have expressed sensitivity in each curve in relation to the calcium concentration which allows a response of 30% of the maximum obtained in the first curve constructed, i.e. 'EC_{30}' = concentration of calcium allowing a response of 30% of initial maximum, obtained by graphical interpolation. This corrects for inter-tissue variations in the absolute size of responses but makes no assumptions about the history of the preparation after the first curve. Thus as desensitization proceeds, the EC_{30} steadily increases and –log EC_{30} falls. For each group of experiments sensitivity is expressed as the arithmetic mean of the –log Ec_{30} values ± S.E. mean.

RESULTS

Concentration/response curves for NA in varying (Ca^{2+}): In the calcium-buffered saline, in which a range of low concentration of (ca^{2+}) could be employed, responses to NA increased from 1 μM (buffer 6) to 0.3mM (buffer 1) (Fig. 1a) (see Table 1 for the concentration of free calcium concentrations in the buffers).

From this data Ca CRCs can be constructed for each NA concentration, (0.1, 0.3, 1.0 and 3μM), the responses increasing with increasing Ca^{2+} CRC shown in figure 3. the sensitivity to NA

calculated in this way approached that found in the 'standard' [Ca^{2+}] of 2.5mM in virgin tissue, but not that found after the 'stabilization procedure' (Fig. 1c).

When the pS2 values (-log EC_{50}) were calculated for the NA concentration/response curves in figure 1a, a decline in sensitivity to noradrenaline was found as [Ca^{2+}]o declined (fig. 1c).

Figure 1
The effects of calcium concentration on responses of the perfused tail artery to noradrenaline. Concentration/response curves for noradrenaline obtained sequentially in the 6 calcium buffer solutions starting in the lowest calcium level, buffer 6, 5, 4, 3, 2, 1 n=12). The data from figure a expressed as free calcium concentration versus the response for four concentration of noradrenaline, 0.1μ M O, 0.3μ 1μM▲, 3μM. ▲The data from figure a expressed as the pD_2 values for noradrenaline versus the free calcium concentration (o). the additional points show the pD_2 obtained in the first noradrenaline concentration/response curve form a separate series of tissues in the presence of the same buffers but with a free calcium concentration of 2.5mM (-) and the stabilized value at 2.5mM calcium after priming with 3μM noradrenaaline (●). Note the direct relationship between calcium level and "sensitivity" to noradrenaline. Bars indicate s.e. of mean.

Figure 2
Composition of the maximum responses obtained in sequences of prolonged, 45min exposures to noradrenaline μ M either in the presence throughout of 2.5mM calcium (●), n=6, or during the construction of "calcium cumulative concentration/response curves" (O, n=12). Time zero marks the end of the inititial equilibration period. Time points plotted indicate the end of an exposure to noradrenaline. Bars indicate S.E. of mean.

Prolonged responses to NA in [Ca^{2+}] free = 2.5mM: Since sensitivity to [Ca^{2+}] was to be assessed, in subsequent experiments, by cumulative CRCs lasting 45mins each, a total of six consecutive 45mins long exposures to NA 3μ M were produces in 2.5mM Ca^{2+} after the 3rd. when this was compared with the NA 3 responses at μ M 2.5mM (Ca^{2+}) obtained in Ca CRCs (see below), the magnitudes of the pressor were similar (FIG 2.). Having established that prolonged responses show desentisation at a constant high level of (ca^{2+}) some factors influencing Ca^{2+} sensitivity during sensitization were then studied.

Ca^{2+} sensitivity of contractile responses to NA and K^+: Arteries were exposed to a concentration of 3μM NA or 100mM K^+ in the presence of a low concentration of free calcium ions in solution (buffer 6). Cumulative increases of [Ca^{2+}] free up to 5.32mM (twice the calcium concentration commonly employed], elicited concentration-dependent contractions.

Six such Ca^{2+} concentration response curves (CCRC) were obtained at 45 minutes intervals. In such a series, the sensitivity of the tissues to [Ca^{2+}] free steadily declined. The maximum responses, the-log (EC^{30}) and the – log (EC^{50}) of the first curves were statistically significantly greater than in the second or subsequent curves. For NA there was a progressive shift which slowed after the 1st CCRC. However, for KCl-induced responses the second and the subsequent curves were not statistically different from each other in any of the above parameters.

(i). ***Noradrenaline***

For NA (3μM), the first CCRC lay further left than the subsequent curves (by 1.13 +s 0.20 log units, n = 6, at the level of –log EC^{30} values) compared with the 2nd. This represents the distance between the means of the individual pairs of EC^{30}s. This first CCEC declined after reaching a peak. It peaked at [Ca^{2+}] free = 0.3 μ M to 1.25mM with 30% (i.e. approximately 50mmHg) of its maximum attained by 30 μ M). The second and subsequent CCRCs showed a more sigmoid correlation of response with log [Ca^{2+}] and responses had not attained a true maximum even at 5mM. The second curve had reached 50mmHg (which is approximately 50% of its "maximum") by 300 μ M; and the third CCRC by about 600 μ M, with the rightward shift slowing down thereafter. For example, the 6th CCRC attained 50mmHg by [Ca^{2+}] = 1mM (Fig. 3).

The peaked first curve makes correction of responses to ringer-tissue variability difficult. Furthermore, comparison of Ca^{2+} sensitivity in different conditions, even in a single tissue, is not straightforward when the "slope" and maximum are changing. We have expressed all pressor responses as a percentage of the 1st maximum to the particular activator, whether this was obtained in a CCRC or during priming.

These 1st maxima were not significantly different between series with the exception of those in the presence of nifedipine. Thus we have compensated only for tissue variability in the height of responses. Ca^{2+} sensitivity was expressed as the concentration producing 30% of this 1st maximum (EC_{30}) (interpolated as –log EC_{30}). Thus, when the maximum changes, this –log EC_{30} value no longer represents a true –log EC_{30} for that curve, but a slightly lower value, exaggerating the extent of desensitization. However even expressed as a percentage of the maximum within each curve, statistically significant desensitization still occurs (data not shown).

The rightward shift (decline) in the sensitivity of tissue to calcium (represented by the EC_{30} values) (Fig. 3) continued with subsequent curves after the second, but to a smaller degree. This rightward shift of the EC_{30}values, expressed as the –log EC_{30}, was used as an index of the fall in sensitivity (Fig. 4a).

(i).Potassium Chloride: High potassium chloride (KCl, 100mM), also produced a peaked first curve which was not repeated in the 2nd or subsequent curves and was, in this respect, similar to NA. However, the [free Ca^{2+}] required for any given response (at 2.5mM Ca^{2+}) with KCl was approximately 10 times higher tha with NA (Fig. 4b). The [free Ca^{2+}] for a 50mMHg response changes from just above 100 μ M in the first curve to 1.25mM in the second curve – a rightward log shift of 0.91 + 0.15 at the level of EC_{30} values, i.e. not significantly different from the situation with NA (1.13 ± 0.2).

Figure 3
Successive calcium cumulative concentration/response curves in the presence of NA 3μM. The first (●), second (●) and sixth (+) in a series are shown. Lines are drawn to indicate diagrammatically the calculation of the EC_{30} based on the initial maximum, n=12. Bars indicate S. E. of mean

Effects of the range of [Ca^{2+}] free used to construct the CRC: The highest concentration of [Ca^{2+}], to which the NA-activated tissue was exposed, affected Ca^{2+} -sensitivity. At the first determination of Ca^{2+} -sensitivity, the maximum concentration usually occurred by 300 μ M Ca^{2+}. When the tissue was exposed to a maximum of 300 μ M Ca^{2+} (buffer 1), desensitization of subsequent CRC's was less than when [Ca^{2+}]o was taken up to 5mM. Another factor enters these later experiments since the tissues were exposed to NA for a shorter time (30min), which might have accounted for less desensitization. However, desensitization was still reduced even when the tissues were left contracted in 300 μ M Ca^{2+} for longer so that the total time for constructing each CCRC was 45min, i.e. the same as for the controls which were exposed to a maximum 5mM Ca^{2+} (Fig. 5a).

Effect of a "priming" contraction to NA in different levels of [free Ca^{2+}] before constructing CCRCs:

(i)"Priming" in Buffer 1 (300 μ M Ca^{2+}): If the tissue were exposed for 5min to NA (3 μ M) in Buffer 1 ("primed") before constructing the first CCRC (up to 5mM Ca^{2+}), desensitization was partially arrested (Fig. 5b). Therefore either priming in 300 μ M Ca^{2+} or taking CRCs to a 'maximum' [Ca^{2+}] of 300 μ M reduced desensitization. Combining the

two was even more effective: the EC_{30} for the 6th CRC was not significantly different from the first (Fig. 5b).

(ii). "Priming" in 2.5mM Ca^{2+}: Priming in 2.5mM Ca^{2+} accelerated desensitization for the 1st two curves although there was some recovery thereafter. This shifted the first curve to the position of the usual second curve. The second curve was shifted to the position of the usual sixth curve (Fig. 5c). Thus while this procedure produced a degree of stability, it did so by making the tissues insensitive to Ca^{2+} by approximately one log unit.

Effect of Bay K 8644 and Nifedipine on the CCRC and on desensitsation

(i). Bay K 8644: Bay K 8644 (0.1 μ M) on its own, unlike the activators NA or KCl, did not produce any change in the baseline, when it was added at any point within the range of [Ca^{2+}] free used. However, it potentiated the first CCRC with NA or KCl (Fig. 4a&b).

Although Bay K 8644 increased the sensitivity to CA^{2+}, desensitization still occurred. However, the EC_{30} in each CCRC indicated greater sensitivity than in the equivalent Bay K 8644-free time control (Fig. 4).

The use of EC_{30} based on initial maximum was not significantly altered by Bay K 8644 (0.1 μ M). The calcium threshold value for a pressor response was alos less when Bay K 8644 was present in the saline. In general, for a given CCRC (1st, 2nd etc) the effect of Bay K 8644 was a parallel displacement to the left, whether the responses were induced by NA or KCl in calcium-buffered saline. Due to the progress of desensitization, addition of Bay K 8644 during construction of a series of curves needs to take into account the time effect. However its potentiating effect can still be clearly seen as is demonstrated in figure 6.

This figure was taken from a study of the effects of oxygen tension on calcium sensitivity, which basically showed that oxygen tension over the range studied had little effect on its own, but that Bay K 8644 potentiated at all oxygen tensions compared with time controls. This was confirmed by repeating the entire protocol at each of these oxygen levels

Figure 4
The desensitization of calcium found in six successive cumulative calcium concentration/response curves with the activators. a.Noradrenaline. b. KCl 100mM (O), n=12. The sensitivity to calcium, expressed as the negativ elogarithm of the concentration producing a response equal to 30% of the maximum in the first curve, is plotted against time. Time zero marks the end of the initial equilibrim period. Time points plotted indicate the end of an exposure to activator. The effects aree shown on Bay K 8644 0.1 μ M, present throughout the experiment (●), n=6, or nifedipine 0μM, added after the sixth curve but before a seventh and eighth (-|-). Bars indicate S.E. of mean

*(ii). **Nifedipine***

Nifedipine (0.1 μ M), attenuated the calcium-dependent contractions of the rat tail artery to noradrenaline and KCl, without altering the baseline. For contractions to noradrenaline, nifedipine decreased sensitivity to Ca^{2+} at the first CCRC compared with untreated controls but thereafter CRC's were not significantly different from their time controls. In contrast, if nifedipine was absent for the first 3 or 6 CRC's, when it was

subsequently added it tended to increase the rate of fall in Ca^{2+} -sensitivity at the next test, though the effect was small (Fig. 7 and fig 4a).

of the concentration producing a response equal to 30% of the maximum in the first curve, is plotted against period. Time points plotted indicates the end of an exposure to noradrenaline. Bar indicate S.E. of mean

Figure 6
The effect of Bay K 8644 0.1μM (●) or nifedipine 0.1μM o(-|-) given during a series of cumulative calcium concentration/reponse curves, (activator noradrenaline 3μ M); drug free controls {O}. The pattern of desensitization, largely unaffected by oxgenation levels was clearly altered by each drug. The sensitivity to calcium, expressed as the negative logarithm of the concentration producing a response equal to 30% of the maximum in the first curve, is plotted against time. Time zero marks the end of the initial equilibration period. Time points plotted indicate the end of a exposure to noradrenaline. Bars indicate S.E. of mean.

Figure 7
The effects of nifedipine 0.1μM (filled symbols) on desensitization during a series of cumulative calcium concentration/response curves, (activator noradrenaline 3 μM); drug free controls (●▲). Nifedipine was present during the whole experiment (-|-) or was added for the 4th and 5th (▲) or the 6th and 7th (●)curves. n=8-12. The sensitivity to calcium, expressed as the negative logarithm of the concentration producing a response equal to 30% of the maximum in the first curve, is plotted against time. Time points plotted indicate the end of an exposure to noradrenaline. Bars indicate S.E. of mean.

Figure 5.
The effects of limiting the calcium concentration during ocnstruction of the curves or of a prior priming response to noradrenaline on calcium sensitivity in six successive cumulative calcium concentration/response curves (activator, noradrenaline 3μM). (a). Control O, maximum calcium tested in curve 5mM, total exposure to noradrenaline 45min, n=12; calcium limited to 0.3mM in curve, total exposure to noradrenaline 30min,□or 45min-|- n=6. (b). Priming response to noradrenaline 3μM, 5min in Ca^{2+} 0.3mM. Control, unprimed, O, n=12; primed, calcium in curve limited to 0.3μ M, ▲ or 5mM, ▲,n=6. (c) Priming response to noradrenaline 3μM, 5min in Ca^{2+} 2.5mM. Control, unprimed, O, n=12; primed, ●, n=6. Calcium in curve limited to 0.3μM for both. The sensitivity to calcium, expressed as the negative logarithm

The inhibition of responses to KCl was more clear-cut. Even after desensitization (6 CCRCs) nifedipine caused a further reduction in sensitivity to Ca^{2+} (fig. 4b). Basing Ca^{2+} sensitivity on EC_{30S} calculated on the initial maximum is not straightforward with nifedipine since it made the 1st maximum significantly smaller. This has the effect of overestimating sensitivity to $[Ca^{2+}]$ after nifedipine when comparing with other situations. This can be taken into account by

(i) Calculating EC_{30} using the actual maximum in each individual curve, or
(ii) Estimating the [Ca^{2+}] which produces a fixed absolute response.

In either case, compared with time controls, nifedipine (0.1μ M) led to a significant decrease ($p<0.05$) in sensitivity of the 1st curve but subsequently this was similar to the time controls (data not shown).

DISCUSSION

The sensitivity to calcium of noradrenaline-induced vasoconstriction of rat tail artery starts at a high value and declines as the experiment progresses. This causes 'desensitization' both to calcium, and when noradrenaline concentration response curves are analysed, to noradrenaline (McGrath et al, 1987a: 1987b). An initial deliberate desensitization procedure leaves a stable but, by definition, less sensitive preparation.

To elucidate the mechanism underlying desensitization, we have examined some factors which influence it, concentrating on the sensitivity to [Ca^{2+}]o since this shows up desensitization even when the response in 2.5mM Ca^{2+} is little altered. It was clear even when constructing the first CCRC that high [Ca^{2+}] was deleterious to the preparation.

Modifying the experimental protocol showed that the highest calcium concentration to which the tissue was exposed affected Ca^{2+} -sensitivity. The higher the [Ca^{2+}]o, the greater the desensitization. This suggests that some form of "Ca overload" may be responsible for the deterioration of responses in this smooth muscle as it does in cardiac muscle (Allen et al, 1985). It has been propose that an internal binding site for calcium on the cell membrane regulates the rate of desensitization (Nastuk and Parsons, 1970; Debassio et al, 1976).

An accumulation of excess free intracellular calcium, in "ca overload", may contribute to desensitization of this tissue via such an internal binding site. This seems to be the case also in guinea-pig ileum where excess calcium accelerates desensitization (Magaribuchi et al, 1973) the different degrees of desensitization produces by priming in different concentrations of ca^{2+} show that priming can produce some stability of subsequent responses but that the remaining level of sensitivity will depend on [Ca^{2+}] both during priming and in the subsequent tests. Therefore priming accelerates desensitization but leaves the tissue relatively insensitive.

This stabilization explains why many workers initially activate their preparation several times until the responses are reproducible (Su et al, 1984; Aoki and Asano, 1986). Clearly, avoidance of high (Ca^{2+}) during priming as well as during subsequent parts of the protocol lead to stable preparations which are significantly less desensitized, since cross-bridge power calculated on the basis that all cross-bridges contribute equally to power production (Niggli, 1999) it is interesting to note that priming in 300μM Ca^{2+} (buffer 1) lead to desensitization. Possibly, on it 1st activation, this lead to Ca overload; but if (Ca^{2+}) is not high the 1st time the tissue is activated, then Ca overload is lessened. Carrying on the hypothesis, second activation produces fewer channel openings. Therefore, if priming is carried out in a protective, low calcium environment, subsequent high sensitivity is ensured, particularly if high calcium is still avoided. Nevertheless if original sensitivity is of interest then perhaps it is best to avoid activation by high (noradrenaline) or high (KCl) altogether. According to such a hypothesis, the site of an initial element is desensitization would lie between receptor activation and the part of the channel responsible for its opening. Some deficit induced here after first activation, e.g. depletion of second messenger substrate, would lead to fewer openings on subsequent activations. The main "damage" however, would be caused by excessive entry.

It was observed in an earlier study that a small concentration of NA (0.3μM), which is one-tenth of the concentration used here, led to less desensitization after the first curve and the subsequent curves stabilized at a higher level of sensitivity than with 3μM NA. This again suggests that extracellular (Ca^{2+}) is not the only factor in desensitization. A further requirement is a sufficiently high stimulus from the activator, possibly by an increased opening of Ca^{2+} channels (either more channels open per unit time or longer open-time per channel).

Bay K 8644 increased Ca^{2+} -sensitivity but did not alter desensitization. This suggests that Bay 8644 sets up a new equlibrium position for tissue responses by allowing further opening of Ca^{2+} channels by the activating stimulus but that at the same time if allows the accumulation of Ca^{2+}, perhaps even enhancing it. This would explain the combination of desensitization within each preparation coupled with potentation of responses relative to time controls.

Nifedipine decreased Ca^{2+} -sensitivity. Interpretation of the effects of nifedipine is not straightforward. Clearly, nifedipine prevented the characteristic 1st responses if given before any activation had occurred. Subsequent responses, however, were no different from time controls (expressed as –log EC_{30}). This suggests that the

initial high sensitivity is produced by a high functional effectiveness of dihydropyridine-sensitive channels and that desensitization in large part consists of the loss of their function.

On its own, the observation that the second CCRC in the presence of nifedipine similar to its time control suggests that after the 1st curve, i.e., in desensitized tissue the main part, if not all, of the response involves Ca^{2+} influx through dihydropyridine-insensitive channels. The acute effect of nifedipine, given after partial desensitization, suggests that in desentised tissue which have not been exposed to nifedipine, some dihydropyridine-sensitive channels remain functional but their contribution to the response is relatively trivial. Thus, dhydropyridine-sensitive channels are significantly involved in the initial response to NA but this element is largely lost after desensitization.

In order to study the calcium sensitivity of noradrenaline-induced vvasoconstriction, it was necessary to select an appropriate concentration of noradrenaline, which could produce well maintained and reproducible responses, and preferably, be sub-maximal. Since desensitization of the tissue combined with normal inter-tissue variation can produce substantial shifts in the noradrenaline pD_2 value across two orders of magnitude, we selected a concentration of 3μM which is maximal in non-desensitized tissue and still approximately 80-90% of maximum in "stabilized" desensitized tissue (data not shown). Since changing the calcium level might be expected to change the efficiency of receptor-contraction coupling, it is possible that the receptor reserve is altered. The noradrenaline concentration/response curves in different calcium buffers are a check on this; they show that there is a decline is the noradrenaline pD_2 value as calcium concentration declines but that, over the calcium concentration range in which the responses are accurately measurable, this effect is not substantial. Furthermore, since the protocol employed a 'priming' exposure to noradrenaline in 2.5mM calcium, it is likely that the sensitivity to noradrenaline shown in the lowest one or two levels of calcium is a slight underestimate. It seems, therefore, that in this tissue and with the receptor system involved, receptor reserve is not greatly influenced within the range of extracellular calcium levels which can sustain responses.

With this particular protocol, responses to norarenaline were highly sensitive to praxosin (results not shown) and are therefore interpreted as being primarily due to a_1-adrenoceptors, although other preparations of the vasculature of the rat tail can show a_2-adrenoceptor-mediated vasoconstriction (Treager et al, 1998; Maurghan and Vigoreaux, 1999): since the $_B$ – adrenergic stimulation can prolong the open time of the L-type Ca^{2+} channels (Templeton et al, 1989).

In conclusion, we have established several factors which influence desensitization in the smooth muscle of rat tail artery. Our results are consistent with a desensitization of Ca^{2+} (2.5 or 5mM) is present during, or subsequent responses. This can be reversed spontaneously by leaving long intervals between tests (6) or can be avoided restricting Ca^{2+} to < 300μM during activation. Some stabilization of sensitivity can be achieved by a ' priming' concentration in Ca^{2+} (300μM or 2.5mM) but this does seem that the initial high sensitivity to Ca^{2+} is a state which is "normal' for this tissue in vitro unless it is exposed to the vigorous insult of a prolonged concentration by a non-physiological concentration of NA at a high [Ca^{2+}].

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