Effects of a water-soluble forskolin derivative (NKH477) and a membrane-permeable cyclic AMP analogue on noradrenaline-induced Ca^{2+} mobilization in smooth muscle of rabbit mesenteric artery

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1 Effects were studied of 6-(3-dimethylaminopropionyl) forskolin (NKH477), a water-soluble forskolin derivative and of dibutyryl-cyclic AMP, a membrane-permeable cyclic AMP analogue on noradrenaline (NA)-induced Ca^{2+} mobilization in smooth muscle strips of the rabbit mesenteric artery. The intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$), isometric force and cellular concentration of inositol 1,4,5-trisphosphate (InsP_3) were measured.

2 NA (10 μM) produced a phasic, followed by a tonic increase in both $[\text{Ca}^{2+}]_i$ and force in a solution containing 2.6 mM Ca^{2+} . NKH477 (0.01–0.3 μM) attenuated the phasic and the tonic increases in both $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA, in a concentration-dependent manner.

3 In Ca^{2+} -free solution containing 2 mM EGTA with 5.9 mM K^+ , NA (10 μM) produced only phasic increases in $[\text{Ca}^{2+}]_i$ and force. NKH477 (0.01 μM) and dibutyryl-cyclic AMP (0.1 mM) each greatly inhibited these increases.

4 NA (10 μM) led to the production of InsP_3 in intact smooth muscle strips and InsP_3 (10 μM) increased Ca^{2+} in Ca^{2+} -free solution after a brief application of Ca^{2+} in β -escin-skinned smooth muscle strips. NKH477 (0.01 μM) or dibutyryl-cyclic AMP (0.1 mM) modified neither the NA-induced synthesis of InsP_3 in intact muscle strips nor the InsP_3 -induced Ca^{2+} release in skinned strips.

5 In Ca^{2+} -free solution, high K^+ (40 and 128 mM) itself failed to increase $[\text{Ca}^{2+}]_i$ but concentration-dependently enhanced the amplitude of the increase in $[\text{Ca}^{2+}]_i$ induced by 10 μM NA with a parallel enhancement of the maximum rate of rise. The extent of the inhibition induced by NKH477 (0.01 μM) or dibutyryl-cyclic AMP (0.1 mM) on the NA-induced $[\text{Ca}^{2+}]_i$ increase was inversely related to the maximum rate of rise of $[\text{Ca}^{2+}]_i$ induced by NA in Ca^{2+} -free solution containing various concentrations of K^+ . These results suggest that the increase in the rate of Ca^{2+} release induced by NA can conceal the inhibitory action on NA-induced Ca^{2+} mobilization of agents that increase cyclic AMP.

6 Repetitive application of 10 μM NA in Ca^{2+} -free solution led to a disappearance of the NA-induced increase in $[\text{Ca}^{2+}]_i$, but NA could again increase $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution after a brief application of Ca^{2+} with 40 mM K^+ (' Ca^{2+} -loading'). The magnitude of this NA-induced increase in $[\text{Ca}^{2+}]_i$ depended on the duration of the Ca^{2+} -loading. With application of dibutyryl-cyclic AMP (0.1 mM) during the Ca^{2+} -loading period, the loading duration required for the restoration of the maximum NA-response was shortened.

7 Cyclopiazonic acid (10 μM , an inhibitor of Ca^{2+} -ATPase at intracellular storage sites) attenuated the inhibitory action of dibutyryl-cyclic AMP on the NA-induced increase in $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution. When NA (10 μM) was applied twice for 30 s with a 10 min interval in Ca^{2+} -free solution, the amplitude of response to the second application was about one third of the first response. With application of 0.1 mM dibutyryl-cyclic AMP during the first application of NA, the increase in $[\text{Ca}^{2+}]_i$ induced by the first application of NA was inhibited, but the response induced by the second was enhanced. These results suggest that dibutyryl-cyclic AMP enhances Ca^{2+} uptake into the NA-sensitive storage sites.

8 We conclude that, in smooth muscle of the rabbit mesenteric artery, agents that increase cyclic AMP inhibit the NA-induced increase in $[\text{Ca}^{2+}]_i$ through an activation of Ca^{2+} uptake into the cellular storage sites.

Keywords: Cyclic AMP; forskolin derivative; vascular smooth muscle; agonist-induced synthesis of inositol 1,4,5-trisphosphate; agonist-induced Ca^{2+} release

Introduction

Activation of β -adrenoceptors in vascular smooth muscle inhibits agonist stimulated contraction by increasing the intracellular concentration of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Bülbring & Tomita, 1987). Forskolin directly activates adenylate cyclase and has been used to study the roles of cyclic AMP in cellular functions in

various types of cell (Seamon & Daly, 1983). A useful recent introduction (Hosono *et al.*, 1990) is the water-soluble forskolin derivative, N,N-dimethyl- β -alanine[3R-(3 α , 4 $\alpha\beta$, 5 β , 6 β , 6 $\alpha\alpha$, 10 α , 10 $\alpha\beta$, 10 $\beta\alpha$)]-5-(acetyloxy)-3-ethenyldodecahydro-10, 10b-dihydroxy-3, 4a, 7, 7, 10a-pentamethyl-1-oxo-1H-naphtho [2,1-b] pyran-6-yl ester hydrochloride (NKH477). This agent, like forskolin, directly activates adenylate cyclase and its potency has been found to be similar to, or greater than that of forskolin (Fujihara *et al.*, 1990). We recently found that, in smooth muscle of the porcine coronary artery, NKH477 is a more potent drug than forskolin for increasing

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the cellular concentration of cyclic AMP (Shafiq *et al.*, 1992).

It has been found that agents that increase cyclic AMP produce vasodilatation with a decrease in $[Ca^{2+}]_i$ in the presence or absence of agonists (Bülbring & Tomita, 1987). This action is caused by an inhibition of Ca^{2+} influx (Muller & van Breemen, 1979; Meisner & van Breemen, 1982), by an activation of Ca^{2+} sequestration (Itoh *et al.*, 1982; Saida & van Breemen, 1984; Eggermont *et al.*, 1988; Furukawa *et al.*, 1988) or by an activation of Ca^{2+} extrusion to the extracellular space (Scheid *et al.*, 1979). It has also been reported that cyclic AMP inhibits agonist-induced increases in $[Ca^{2+}]_i$ by reducing the synthesis of inositol 1,4,5-trisphosphate (InsP₃, Watson *et al.*, 1984; Takenawa *et al.*, 1986; Madison & Brown, 1988) or through the phosphorylation of the InsP₃ receptor (Supattapone *et al.*, 1988; Yamamoto *et al.*, 1989). However, the mechanism underlying the inhibition of agonist-induced Ca^{2+} mobilization by agents which increase cyclic AMP has not been well characterized in vascular smooth muscle.

To investigate this, we studied the effects of NKH477 and dibutyl-cyclic AMP on the increases in $[Ca^{2+}]_i$ and force and InsP₃ synthesis induced by NA in smooth muscle strips of the rabbit mesenteric artery. The effects of NKH477 and dibutyl-cyclic AMP on InsP₃-induced Ca^{2+} release were also studied in β -escin-skinned smooth muscle strips.

Methods

Male albino rabbits, weighing 1.9–2.3 kg, were anaesthetized with pentobarbitone sodium (40 mg kg⁻¹, i.v.), and then exsanguinated. The third branch of the mesenteric artery was immediately excised and cleaned by removal of connective tissue in Krebs solution at room temperature.

Simultaneous recording of Ca^{2+} and force

To permit simultaneous recording of $[Ca^{2+}]_i$ and isometric force, fine circularly-cut strips (0.3–0.5 mm length, 0.04–0.05 mm width, 0.02–0.03 mm thickness) were prepared as described previously (Itoh *et al.*, 1983). Endothelial cells were removed by gentle rubbing of the internal surface of the vessels with small knives. The absence of endothelial cells was confirmed by the inability of acetylcholine (1 μ M) to cause relaxation during contractions induced by 10 μ M NA, as described previously (Itoh *et al.*, 1992). The strip was transferred into a chamber of 0.3 ml volume and mounted horizontally on an inverted-microscope (Diaphot TMD with special optics for epifluorescence, Nikon). The resting force was adjusted so as to obtain a maximal contraction in 128 mM K⁺.

To load Fura-2 into the smooth muscle cells of the strip, 1 μ M acetoxymethyl ester of Fura-2 (Fura-2 AM) dissolved in dry dimethyl sulphoxide (1 mM stock solution) was applied for 1 h in Krebs solution at room temperature (23–25°C). The strip was moved to the centre of the field and a mask (0.04 mm square) placed in an intermediate image plane of the microscope to reduce background fluorescence. The Fura-2 fluorescence emission at 510 nm (passed through an interference filter centred at 510 nm with a full width at half transmission of 20 nm) was passed through the objective lens (20 x fluor, Nikon) and collected with a photomultiplier tube (R928, side-on type, Hamamatsu Photonics, Japan) via a dichroic mirror (DM-400, Nikon) which was substituted for the photochanger in a Nikon Diaphot-TMD microscope. Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm) were applied by a spectro-fluorimeter (Spex, NJ, U.S.A.) and the data were analyzed with customized software provided by Spex (DM-3000 CM).

The ratio of the Fura-2 fluorescence intensities excited by 340 and 380 nm was calculated after subtraction of the background fluorescence. Background fluorescence (including the autofluorescence of the strip) excited by 340 and 380 nm

u.v.-light was measured after the experiment following application of a solution containing 50 μ M ionomycin, 20 mM MnCl₂, 110 mM KCl and 10 mM 3-(N-morpholino) propanesulphonic acid (MOPS) (pH 4.8). Under these conditions, the background fluorescence intensity was 10–15% of the Fura-2 signals in smooth muscle strips at either excitation wavelength. Cytosolic Ca^{2+} concentrations were calculated by the formula described by Grynkiewicz *et al.* (1985) and using *in vitro* calibration (Poenie *et al.*, 1986; Becker *et al.*, 1989). The ratio of maximum (F_{max}) to minimum fluorescence (F_{min}) was determined in the calibration solution after subtraction of background excited by either 340 or 380 nm, and the 380 signals of Fura-2 were assumed to decrease by 15% in the cell due to the possible intracellular viscosity effects of the dye (Poenie *et al.*, 1986; Becker *et al.*, 1989). The K_d value for Fura-2 was assumed to be 200 nM (Becker *et al.*, 1989). To enable comparison of the effects of drugs on intact and skinned smooth muscle strips and to prevent the extrusion of Fura-2 (Roe *et al.*, 1990), the present experiments were performed at room temperature.

Experiment on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were prepared using β -escin (Itoh *et al.*, 1992). The methods used to prepare the skinned muscle strips and the composition of the solutions used have been described elsewhere (Itoh *et al.*, 1986; 1992). To enable measurement of Ca^{2+} release from intracellular storage sites, 0.3 μ M Ca^{2+} buffered with 4 mM EGTA was applied for 5 min (to load Ca^{2+} into the storage sites). Ca^{2+} was then removed from the bathing solution by the application of Ca^{2+} -free solution containing 4 mM EGTA for 0.5 min. A solution containing 50 μ M EGTA and 2 μ M Fura-2 was then applied for 5 min. Finally, 10 μ M InsP₃ was applied for 2 min in a solution containing 50 μ M EGTA and 2 μ M Fura-2.

Measurement of InsP₃

Endothelium-denuded strips (15 mm length, 0.3–0.5 mm width, 0.1 mm thickness) were equilibrated for over 2 h in Krebs solution at 32°C. After this, the strips were transferred to Ca^{2+} -free solution containing 2 mM EGTA for 2 min and then 10 μ M NA was applied for 10 s. NKH477 or dibutyl-cyclic AMP was given as a pretreatment for 5 min in Krebs solution, for 2 min in Ca^{2+} -free solution and throughout the application of NA. The reaction was stopped by the addition of a large amount of ice-cold 8% trichloroacetic acid and the strips were homogenized. The homogenate was centrifuged and the supernatant fraction treated with water-saturated ether three times and assayed with a radioimmunoassay kit from Amersham International, as described previously (Itoh *et al.*, 1992).

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na⁺ 137.4, K⁺ 5.9, Mg²⁺ 1.2, Ca^{2+} 2.6, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134.2 glucose 11.5. The concentration of K⁺ was modified by replacing NaCl with KCl, isosmotically. To prevent both NA outflow from sympathetic nerve terminals and β -adrenoceptor stimulation by exogenously-applied NA, 3 μ M guanethidine and 0.3 μ M propranolol were present in the Krebs solution throughout the experiment. Ca^{2+} -free solution was made by substituting an equimolar concentration of MgCl₂ for CaCl₂ and adding 2 mM EGTA. The solutions were bubbled with 95% O₂ and 5% CO₂ and their pH was maintained at 7.3–7.4.

The calibration solution for Ca^{2+} measurement in intact strips contained 11 mM EGTA, 110 mM KCl, 1 mM MgCl₂, 2 μ M Fura-2 and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (pH 7.1) with or without 11 mM CaCl₂.

For experiments on skinned muscle, the composition of the relaxing solution was: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N,N'*-bis-(2-ethanesulphonic acid) (PIPES), 5.1 mM $\text{Mg}(\text{MS})_2$, 5.2 mM ATP, 5 mM phosphocreatine and 4 mM ethyleneglycol-bis-(β -aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA). To enable measurement of Ca^{2+} release from skinned strips, the concentration of EGTA was reduced to 50 μM and 2 μM Fura-2 was added. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $\text{Ca}(\text{MS})_2$ to 4 mM EGTA, based on the calculation reported previously (Itoh *et al.*, 1986). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength standardized at 0.2 M by changing the amount of KMS added.

Drugs

Drugs used were Fura-2, Fura-2 AM, InsP_3 , EGTA, PIPES, HEPES and MOPS (Dojin, Japan), NA and cyclopiazonic acid (CPA), dibutylrly-cyclic AMP and β -escin (Sigma), guanethidine (Tokyo Kasei, Japan), ATP (Na salt; Kojin, Japan) and propranolol (Nacalai, Japan). *N,N*-dimethyl- β -alanine [3R-(3 α , 4 α , 5 β , 6 β , 6 α , 10 α , 10 β , 10 $\beta\alpha$)]-5 (acetyloxy)-3-ethenyl-dodecahydro-10, 10b-dihydroxy-3, 4a, 7, 7, 10a-pentamethyl-1-oxo-1H-naphthol[2,1-b]pyran-6-yl ester hydrochloride (NKH477) was kindly provided by Nippon Kayaku Co., Ltd., (Tokyo, Japan).

Statistics

The values recorded were expressed as mean $\pm$ s.d. and statistical significance was determined by a paired or unpaired Student's *t* test. Probabilities less than 5% ($P < 0.05$) were considered significant.

Results

Effects of NKH477 on increases in $[\text{Ca}^{2+}]_i$ and force induced by NA in the presence of Ca^{2+}

Figure 1a shows traces of the effect of NKH477 on the increases in $[\text{Ca}^{2+}]_i$ (i) and force (ii) induced by 10 μM NA in Krebs solution (containing 2.6 mM Ca^{2+}). NA (10 μM) was applied for 2 min at 30 min intervals. Under these conditions, the resting $[\text{Ca}^{2+}]_i$ was 84.0 ± 3.7 nM ($n = 4$). Application of 10 μM NA produced a phasic, then a tonic increase in $[\text{Ca}^{2+}]_i$ and force. The phasic increases in $[\text{Ca}^{2+}]_i$ and force took their levels to 243.5 ± 17.2 nM and 10.4 ± 4.6 mg, respectively and the tonic levels reached (measured 2 min into the application) were 133.2 ± 15.7 nM and 9.6 ± 4.7 mg, respectively ($n = 4$). NKH477 (0.01–0.3 μM) produced concentration-dependent attenuations of the NA-induced phasic (Figure 1b(i)) and tonic (Figure 1b(ii)) increases in $[\text{Ca}^{2+}]_i$ and force, the two variables being affected in parallel.

In smooth muscle of the rabbit mesenteric artery, the NA-induced phasic increase in $[\text{Ca}^{2+}]_i$ is thought to be caused by Ca^{2+} release from its intracellular storage sites (Ito *et al.*, 1991; Itoh *et al.*, 1992). Since NKH477 is a potent activator of adenylate cyclase (Shafiq *et al.*, 1992), these results suggest that NKH477 inhibits NA-induced Ca^{2+} release through an activation of cyclic AMP-dependent mechanisms.

Effects of NKH477 and dibutylrly-cyclic AMP on increases in $[\text{Ca}^{2+}]_i$ and force induced by NA in Ca^{2+} -free solution containing 5.9 mM K^+

To investigate more precisely the effect of agents that increase cyclic AMP on NA-induced Ca^{2+} release, the effects were studied of NKH477 and dibutylrly-cyclic AMP on the increases in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+} -free solution containing 2 mM EGTA with 5.9 mM K^+ . NA (10 μM) was applied for 2 min in Ca^{2+} -free solution 2 min

after removal of Ca^{2+} , the strips being kept in Krebs solution (containing 2.6 mM Ca^{2+}) for 25 min between the tests. Following 2 min removal of Ca^{2+} , $[\text{Ca}^{2+}]_i$ rapidly decreased to 72.0 ± 6.2 nM ($n = 4$) and an application of NA (10 μM) produced a transient increase in $[\text{Ca}^{2+}]_i$ (to 165.8 ± 23.5 nM) and in force (to 6.2 ± 1.9 mg) ($n = 4$). NKH477 (0.01 and 0.1 μM) did not change the resting $[\text{Ca}^{2+}]_i$ significantly but concentration-dependently inhibited the increases in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA. Dibutylrly-cyclic AMP (0.1 mM) also inhibited the increases in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+} -free solution containing 5.9 mM K^+ without a significant change in the resting concentration of $[\text{Ca}^{2+}]_i$.

In Ca^{2+} -free solution containing 5.9 mM K^+ , caffeine (5 mM) transiently increased both $[\text{Ca}^{2+}]_i$ and force, the maximum increases being to 298.3 ± 71.2 nM and 5.2 ± 1.1 mg, respectively ($n = 4$). Neither NKH477 (0.01 μM) nor dibutylrly-cyclic AMP (0.1 mM) had any effect on the caffeine-induced increases in $[\text{Ca}^{2+}]_i$ and force.

Effects of NKH477 on NA-induced InsP_3 production

In smooth muscle of the rabbit mesenteric artery, InsP_3 is thought to be a possible second messenger in the NA-induced release of Ca^{2+} from intracellular storage sites (Hashimoto *et al.*, 1986; Ito *et al.*, 1991; Itoh *et al.*, 1992). To investigate the sites involved in the inhibitory actions of NKH477 and dibutylrly-cyclic AMP on the NA-induced increase in $[\text{Ca}^{2+}]_i$, the effects of these agents on NA-induced InsP_3 synthesis were studied in Ca^{2+} -free solution containing 5.9 mM K^+ . NA (10 μM) was applied for 2 min after 2 min removal of Ca^{2+} . Under these conditions, NA (10 μM) transiently increased the amount of InsP_3 at 10 s, the effect decaying within 30 s (Itoh *et al.*, 1992). The effects of NKH477 (0.01 μM) or dibutylrly-cyclic AMP (0.1 mM) on NA-induced synthesis of InsP_3 were therefore measured 10 s after the application of NA.

The concentration of InsP_3 was 5.1 ± 3.1 pmol mg^{-1} protein ($n = 4$) before, and 13.6 ± 3.9 pmol mg^{-1} protein ($n = 4$) 10 s after application of 10 μM NA. Neither NKH477 (0.01 μM) nor dibutylrly-cyclic AMP (0.1 mM) had any effect on this NA-induced InsP_3 synthesis (14.7 ± 0.5 pmol mg^{-1} protein in the presence of 0.01 μM NKH477, $n = 4$; 18.2 ± 2.8 pmol mg^{-1} protein in the presence of 0.1 mM dibutylrly-cyclic AMP, $n = 4$).

Effects of NKH477 and dibutylrly-cyclic AMP on InsP_3 -induced Ca^{2+} release in β -escin-treated skinned smooth muscle

To investigate further the sites involved in the inhibitory actions of NKH477 and dibutylrly-cyclic AMP on the NA-induced increase in $[\text{Ca}^{2+}]_i$, the effects of NKH477 and dibutylrly-cyclic AMP were observed on the InsP_3 -induced Ca^{2+} release in β -escin-treated skinned smooth muscle strips. After the strips had been skinned by an application of 25 μM β -escin for 30 min, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 5 min to load Ca^{2+} into the storage sites (' Ca^{2+} -loading') and Ca^{2+} -free solution containing 4 mM EGTA was applied for 0.5 min to remove Ca^{2+} from the solution. Finally, 10 μM InsP_3 was applied for 2 min in Ca^{2+} -free solution containing 50 μM EGTA and 2 μM Fura-2. Under these conditions, the resting $[\text{Ca}^{2+}]_i$ was 83.7 ± 5.6 nM ($n = 4$) and InsP_3 (10 μM) increased $[\text{Ca}^{2+}]_i$ to 146.6 ± 24.6 nM ($n = 4$), possibly due to release of Ca^{2+} from its storage sites. Neither NKH477 (0.1 μM) nor dibutylrly-cyclic AMP (0.1 mM) modified the InsP_3 -induced Ca^{2+} release when each drug was applied separately in Ca^{2+} -free solution after Ca^{2+} -loading (0.92 ± 0.11 times control in the presence of 0.01 μM NKH477 and 1.06 ± 0.27 times control in the presence of 0.1 mM dibutylrly-cyclic AMP, $n = 6$). However, under these conditions, NKH477 (0.01 μM) and dibutylrly-cyclic AMP (0.1 mM)

Figure 1 Concentration-dependent effects of NKH477 on increases in $[Ca^{2+}]_i$ and force induced by noradrenaline (NA) in the presence of 2.6 mM Ca^{2+} . (a) (i) $[Ca^{2+}]_i$ and (a) (ii) force. NA (10 μM) was applied for 2 min (indicated by bar) at 30 min intervals. NKH477 (0.01 and 0.1 μM) was applied for 5 min before and during application of NA. The results illustrated were obtained from a single smooth muscle strip and are overlaid in the figure. (b) (i) Concentration-dependent effects of NKH477 on phasic increase in $[Ca^{2+}]_i$ (○) and force (Δ) induced by 10 μM NA. The amplitude of the increase in $[Ca^{2+}]_i$ induced by NA is expressed as ' $\Delta [Ca^{2+}]_i$ ' (the peak minus the resting $[Ca^{2+}]_i$). The maximum force induced by 10 μM NA in the absence of NKH477 was normalized as a relative force of 1.0. (b) (ii) The effects on NA-induced tonic increase in $[Ca^{2+}]_i$ (○) and force (Δ). The tonic responses were measured 2 min after the application of 10 μM NA and the force induced by 10 μM NA in the absence of NKH477 was normalized as a relative force of 1.0. Results shown are each the mean $\pm$ s.d. of 4 observations. *Statistically significant difference from the value obtained in the absence of NKH477 ($P < 0.05$).

each inhibited the contraction induced by 0.3 μM Ca^{2+} , as reported previously for smooth muscle strips of the porcine coronary artery (Shafiq *et al.*, 1992). These results suggest that cyclic AMP does indeed have some inhibitory action on the contractile proteins in β -escin skinned smooth muscle strips.

Effects of NKH477 and dibutyryl-cyclic AMP on increase in $[Ca^{2+}]_i$ induced by NA in Ca^{2+} -free solution containing high K^+

The magnitude of the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+} -free solution may depend on the rates of Ca^{2+} release and Ca^{2+} removal in the presence of NA in smooth muscle cells. If this were so, it would be expected that, when Ca^{2+} removal mechanisms are activated by NKH477 or dibutyryl-cyclic AMP, the size of the NA-induced increase in $[Ca^{2+}]_i$ would be reduced. Moreover, the inhibitory action of NKH477 or dibutyryl-cyclic AMP on the NA-induced $[Ca^{2+}]_i$ increase might be concealed as the rate of the NA-induced Ca^{2+} release increases. We have previously found that K^+ concentration-dependently enhances the NA-induced increase in $[Ca^{2+}]_i$ without any increase in the NA-induced $InsP_3$ synthesis (Itoh *et al.*, 1992). To test the hypothesis, we studied the effects of NKH477 and dibutyryl-cyclic AMP on the increase in $[Ca^{2+}]_i$ induced by 10 μM NA in Ca^{2+} -free solution containing various concentrations of high K^+ .

Ca^{2+} -free solution containing high K^+ (40 or 128 mM) was applied 1 min after application of Ca^{2+} -free solution containing 2 mM EGTA with 5.9 mM K^+ . High K^+ itself failed to increase either $[Ca^{2+}]_i$ or force, but concentration-dependently enhanced the NA-induced increase in $[Ca^{2+}]_i$ with a corresponding increase in the maximum value of the rate of rise. NKH477 (0.01 μM) greatly inhibited the NA-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+} -free solution containing 5.9 mM K^+ , but the inhibition was attenuated in Ca^{2+} -free solution containing 40 mM K^+ and abolished in Ca^{2+} -free solution containing 128 mM K^+ (Figure 2). Dibutyryl-cyclic AMP (0.1 mM) also inhibited the increases in $[Ca^{2+}]_i$ (0.14 ± 0.02 times control, $n = 4$) and force (0.18 ± 0.14 times control, $n = 4$) in Ca^{2+} -free solution containing 5.9 mM K^+ , but the inhibition was attenuated in Ca^{2+} -free solution containing 40 mM K^+ (0.58 ± 0.08 times control for $[Ca^{2+}]_i$ and 0.61 ± 0.19 times control for force, $n = 4$) and in Ca^{2+} -free solution containing 128 mM K^+ (0.75 ± 0.02 times control for $[Ca^{2+}]_i$ and 0.86 ± 0.14 times control for force, $n = 4$).

Figure 3 shows the relationship between the maximum rate of rise of $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ in the presence of 10 μM NA in Ca^{2+} -free solution containing various concentrations of K^+ (5.9–128 mM). The relationship between the two was, to a first approximation, described by a linear function (correlation coefficient, $r = 0.98$). NKH477 (0.01 μM , Figure 3a) and dibutyryl-cyclic AMP (0.1 mM, Figure 3b) each attenuated both the maximum rate of rise of $[Ca^{2+}]_i$

Figure 2 Effects of NKH477 on increases in $[\text{Ca}^{2+}]_i$ and force induced by noradrenaline (NA) in Ca^{2+} -free solution containing 2 mM EGTA with high K^+ . NA (10 μM) was applied for 2 min in Ca^{2+} -free solution after a 2 min removal of Ca^{2+} . Ca^{2+} -free solution containing high K^+ (40 or 128 mM) was applied 1 min after an application of Ca^{2+} -free solution containing 2 mM EGTA with 5.9 mM K^+ . NA (10 μM) was applied for 2 min in Ca^{2+} -free solution containing various concentrations of K^+ . NKH477 (0.01 μM) was applied for 3 min in Krebs solution, for 2 min in Ca^{2+} -free solution and throughout the application of 10 μM NA. (a) Shows actual traces of $[\text{Ca}^{2+}]_i$ (i) and force (ii). (1) Control; (2) in the presence of 0.01 μM NKH477. (b) The effects of 0.01 μM NKH477 on increase in $[\text{Ca}^{2+}]_i$ (i) and relative force (ii) induced by 10 μM NA in Ca^{2+} -free solution containing various concentrations of K^+ (5.9, 40 and 128 mM). Open columns: control; hatched columns: in the presence of 0.01 μM NKH477. Results shown are each the mean with s.d. of 3 observations.

and the increase in $[\text{Ca}^{2+}]_i$ induced by 10 μM NA in Ca^{2+} -free solution containing 5.9 mM or 40 mM K^+ , but not 128 mM K^+ .

High K^+ depolarizes the membrane in smooth muscle cells of the rabbit mesenteric artery, in a concentration-dependent manner (Itoh *et al.*, 1983). Thus, it would be expected that K^+ would attenuate the inhibitory actions of NKH477 and dibutyl-cyclic AMP on the NA-induced increase in $[\text{Ca}^{2+}]_i$ through its membrane depolarizing action. To examine this possibility, the effects were studied of NKH477 and dibutyl-cyclic AMP on the NA-induced $[\text{Ca}^{2+}]_i$ increase in Ca^{2+} -free solution containing 128 mM K^+ under conditions in which the amount of stored Ca^{2+} in the cells was varied. Following repetitive application of 10 μM NA in Ca^{2+} -free solution containing 2 mM EGTA with 5.9 mM K^+ , the muscle strips were incubated with Ca^{2+} -free solution containing 5.9 mM K^+ for 5 min. Then, 2.6 mM Ca^{2+} with 40 mM K^+ was applied for various times (' Ca^{2+} -loading') and Ca^{2+} -free solution containing 5.9 mM K^+ subsequently applied for 9 min. Finally, NA (10 μM) was applied after 1 min of an application of Ca^{2+} -free solution containing 128 mM K^+ (Figure 4a). The amplitude of the NA-induced response increased in proportion with the duration of the Ca^{2+} -loading for times up to 60 s. Either NKH477 (0.01 μM) or dibutyl-cyclic AMP (0.1 mM) was applied in Ca^{2+} -free solution after the Ca^{2+} -loading period. Both NKH477 and dibutyl-cyclic AMP inhibited the increase in $[\text{Ca}^{2+}]_i$ induced by 10 μM NA

in Ca^{2+} -free solution containing 128 mM K^+ when the time set for the Ca^{2+} -loading was shorter than 60 s (Figure 4b). These results suggest that the membrane depolarization induced by high K^+ may not be responsible for the attenuation of the inhibitory action of NKH477 or dibutyl-cyclic AMP on the NA-induced $[\text{Ca}^{2+}]_i$ increase which occurs in the presence of high K^+ .

Since K^+ replaced an equimolar concentration of Na^+ in the high K^+ solution, a lack of Na^+ in the Ca^{2+} -free solution might have influenced the effect of NKH477 and dibutyl-cyclic AMP. The effects of NKH477 were therefore observed on the increases in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+} -free, Na^+ -deficient solution. NaCl (121.9 mM) was replaced by Tris-Cl (containing 15.5 mM Na^+). When the Ca^{2+} -free, Na^+ -deficient solution was applied 1 min after removal of Ca^{2+} by Ca^{2+} -free solution containing 5.9 mM K^+ with 137.4 mM Na^+ , the Na^+ -deficient solution itself failed to increase either $[\text{Ca}^{2+}]_i$ or force but did somewhat enhance the increases in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA. The peak values for the effects of NA (10 μM) on $[\text{Ca}^{2+}]_i$ and force were respectively 158.2 ± 24.9 nM and 4.2 ± 1.0 mg in Ca^{2+} -free solution containing 137.4 mM Na^+ , and 251.2 ± 31.1 nM and 7.9 ± 1.8 mg in Ca^{2+} -free solution containing 15.5 mM Na^+ ($n = 4$). NKH477 (0.01 μM) inhibited the increase in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+} -free, Na^+ -deficient solution (116.4 ± 10.0 nM and 1.1 ± 0.4 mg in the presence of 0.01 μM NKH477, $n = 4$).

Figure 3 The relationship between the maximum rate of rise of $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by noradrenaline (NA) in Ca^{2+} -free solution containing various concentrations of K^+ in the absence or presence of NKH477 (a) or dibutylrlyl cyclic AMP (b). NA ($10\ \mu M$) was applied for 2 min in Ca^{2+} -free solution containing 5.9 mM ($\bullet$, $\circ$), 40 mM ($\blacktriangle$, $\triangle$) or 128 mM ($\blacksquare$, $\square$) K^+ in the absence (solid symbols) or presence (open symbols) of $0.01\ \mu M$ NKH477 or 0.1 mM dibutylrlyl-cyclic AMP. The amplitude of the maximum increase in $[Ca^{2+}]_i$ induced by NA is expressed as $\Delta [Ca^{2+}]_i$. Results shown are each the mean with s.d. of 3 observations.

Effects of dibutylrlyl-cyclic AMP on Ca^{2+} uptake into the NA-sensitive storage sites

The effects of dibutylrlyl-cyclic AMP were studied on Ca^{2+} uptake into the storage sites using a protocol similar to that described in Figure 4a, except that the dibutylrlyl-cyclic AMP was applied during the Ca^{2+} -loading period (Figure 5a). When Ca^{2+} was applied simultaneously with 0.1 mM dibutylrlyl-cyclic AMP, the amplitude of the NA-induced increase in $[Ca^{2+}]_i$ was enhanced when the duration for Ca^{2+} -loading was set within 20 s ($P < 0.05$, paired t test, $n = 4$), but the maximum NA-induced response obtained with a 1 min application of 2.6 mM Ca^{2+} was not affected (Figure 5b). These results suggest that dibutylrlyl-cyclic AMP may increase not Ca^{2+} influx, but rather the rate of Ca^{2+} uptake into the

Figure 4 Effects of NKH477 and dibutylrlyl-cyclic AMP (db-Cyclic AMP) on increase in $[Ca^{2+}]_i$ induced by noradrenaline (NA) in Ca^{2+} -free solution containing 128 mM K^+ under conditions in which the amount of stored Ca^{2+} in the cells was varied. (a) Shows the experimental protocol. The muscle strips were initially kept in Krebs solution, followed by a 5 min exposure to Ca^{2+} -free solution containing 5.9 mM K^+ . NA ($10\ \mu M$) was then repetitively applied before the experiment started. Ca^{2+} (2.6 mM) with 40 mM K^+ was applied for various durations (t(s)) and then Ca^{2+} -free solution containing 5.9 mM K^+ applied for 9 min. Finally, $10\ \mu M$ NA was applied for 2 min after 1 min application of Ca^{2+} -free solution containing 128 mM K^+ . (b) NA-induced increase in $[Ca^{2+}]_i$ as a function of the duration of the Ca^{2+} -application. The maximum increase in $[Ca^{2+}]_i$ induced by $10\ \mu M$ NA following a 120 s application of 2.6 mM Ca^{2+} was normalized as a relative increase in $[Ca^{2+}]_i$ of 1.0. ($\bullet$): Control. Either db-cyclic AMP ($0.1\ mM$, $\circ$) or NKH477 ($0.01\ \mu M$, $\triangle$) was applied before and during application of $10\ \mu M$ NA in Ca^{2+} -free solution, as indicated in (a). Results shown are each the mean of 4 observations $\pm$ s.d. shown by a vertical bar.

NA-sensitive storage sites under the present experimental conditions.

The effect of NKH477 on the rate of Ca^{2+} uptake was not examined since the inhibitory action of NKH477 ($0.01\ \mu M$) on the NA-induced increase in $[Ca^{2+}]_i$ continued for more than 30 min after washout.

To study further the effects of dibutylrlyl-cyclic AMP on Ca^{2+} uptake into the NA-sensitive storage sites, the effects of dibutylrlyl-cyclic AMP were observed on the increase in $[Ca^{2+}]_i$ induced by repetitive application of NA in Ca^{2+} -free solution containing 5.9 mM K^+ . Following 2 min removal of Ca^{2+} , NA ($10\ \mu M$) was applied twice for 30 s with a 10 min interval in Ca^{2+} -free solution (Figure 6a). Under these conditions, the amplitude of the increase in $[Ca^{2+}]_i$ induced by the second application of $10\ \mu M$ NA was one third that induced by the first (0.32 ± 0.09 times, $n = 4$). When 0.1 mM dibutylrlyl-cyclic AMP was given during the first application of $10\ \mu M$ NA, the amplitude of the first NA-response was reduced (0.30 ± 0.05 times the first NA-response without application of dibutylrlyl-cyclic AMP, $n = 4$) but the second NA-response was increased (2.09 ± 0.32 times the second NA-response without application of dibutylrlyl-cyclic AMP, $n = 4$). In accordance with the previous findings based on mechanical responses (Itoh *et al.*, 1983; 1985), these results suggest that some of the Ca^{2+} released induced by the first application of NA undergoes re-uptake into the storage sites and dibutylrlyl-cyclic AMP may enhance this Ca^{2+} uptake.

Figure 5 Effects of dibutyl-cyclic AMP (db-cyclic AMP) applied during pre-incubation with Ca^{2+} on increase in $[\text{Ca}^{2+}]_i$ induced by noradrenaline (NA) in Ca^{2+} -free solution containing 128 mM K^+ . (a) Shows the experimental protocol. Db-cyclic AMP (0.1 mM, $\square$) was applied for 3 min in Ca^{2+} -free solution and throughout the application of Ca^{2+} , as indicated in (a). The rest of the experimental protocol was as described in Figure 4a. (b) NA-induced increase in $[\text{Ca}^{2+}]_i$ (normalized as in Figure 4b) as a function of the duration of the Ca^{2+} -application. Control responses shown in Figure 5b ($\bullet$) are reproduced from Figure 4b. Results shown are each the mean of 4 observations with s.d. shown by a vertical bar.

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, has been reported to be a highly selective inhibitor of Ca^{2+} -ATPase in the sarcoplasmic reticulum (SR) of skeletal muscle (Seidler *et al.*, 1989; Kurebayashi & Ogawa, 1991). To study further the effects of dibutyl-cyclic AMP on Ca^{2+} uptake into the NA-sensitive storage sites, the effects of dibutyl-cyclic AMP were observed on the increase in $[\text{Ca}^{2+}]_i$ induced by repetitive application of NA in the presence of 10 μM CPA. Following 2 min removal of Ca^{2+} , NA (10 μM) was applied twice for 30 s with a 10 min interval in Ca^{2+} -free solution in the presence or absence of 10 μM CPA. When used, CPA (10 μM) was applied 90 s before the first application of 10 μM NA and was present throughout the rest of the experiment. CPA (10 μM) abolished the increase in $[\text{Ca}^{2+}]_i$ induced by the second application of NA without any change in the amplitude of the first NA-response (Figure 6b (ii)). These results suggest that CPA inhibits the re-uptake of Ca^{2+} into the storage sites during the Ca^{2+} mobilization induced by the first application of NA. Following a 60 min washout of CPA with Krebs solution, the increases in $[\text{Ca}^{2+}]_i$ induced by repetitive application of 10 μM NA recovered completely. When 0.1 mM dibutyl-cyclic AMP was applied during the first application of 10 μM NA in the presence of 10 μM CPA, the inhibitory action of dibutyl-cyclic AMP on the increase in $[\text{Ca}^{2+}]_i$ induced by the first application of NA was attenuated (0.60 ± 0.05 times the control in the presence of 10 μM CPA, $n = 4$ and see also Figure 6a(ii) for the action of dibutyl-cyclic AMP in the absence of CPA) and there was no response to the second application of NA (Figure 6b(iii)).

Discussion

In the present experiments, NA produced a phasic followed by a tonic increase in both $[\text{Ca}^{2+}]_i$ and force in the presence of 2.6 mM Ca^{2+} in smooth muscle of the rabbit mesenteric

Figure 6 Effects of dibutyl-cyclic AMP (db-cyclic AMP) on increase in $[\text{Ca}^{2+}]_i$ induced by repetitive application of noradrenaline (NA) in Ca^{2+} -free solution containing 5.9 mM K^+ in the absence (a) or presence (b) of cyclopiazonic acid (CPA). Following 2 min application of the Ca^{2+} -free solution, NA (10 μM) in Ca^{2+} -free solution was applied twice for 30 s with a 10 min interval. In (a) (i) control. (a) (ii) DB-cyclic AMP (0.1 mM) was applied for 2 min in Ca^{2+} -free solution and throughout the first application of NA. Between traces a(i) and a(ii), Krebs solution (containing 2.6 mM Ca^{2+}) was applied for 25 min to ensure reproducible NA responses in Ca^{2+} -free solution. In (b): b(i) control; b(ii) in the presence of 10 μM CPA. CPA (10 μM) was applied 1 min after application of Ca^{2+} -free solution and was present for the rest of the experiment. Between traces b(i) and b(ii), Krebs solution containing 2.6 mM Ca^{2+} was applied for 25 min to ensure reproducible NA responses in Ca^{2+} -free solution. After the procedure shown in trace b(ii), CPA was washed out by Krebs solution and the muscle strip kept for 60 min in Krebs solution to ensure complete recovery of the NA-responses in Ca^{2+} -free solution. b(iii) Db-cyclic AMP (0.1 mM) was applied 1 min after application of Ca^{2+} -free solution and throughout the first application of NA. Results shown are representative of three other experiments.

artery. The NA-induced phasic increases in $[\text{Ca}^{2+}]_i$ and force persisted in Ca^{2+} -free solution, suggesting that these phasic responses are probably due to a release of Ca^{2+} from its intracellular storage sites. We have previously reported that, in smooth muscle of the rabbit mesenteric artery, NA activates the synthesis of $[\text{H}^3]$ -InsP₃ through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂), and InsP₃ releases ^{45}Ca from intracellular storage sites (Hashimoto *et al.*, 1986). In the present experiments, we partly confirmed these observations in that NA increased the synthesis of InsP₃ in intact smooth muscle strips (measured by radioimmunoassay)

and InsP_3 released Ca^{2+} from the cellular storage sites in β -escin-treated skinned smooth muscle (measuring by Fura-2 fluorescence). These results suggest that InsP_3 is a second messenger in the NA-induced release of Ca^{2+} from intracellular storage sites in smooth muscle of the rabbit mesenteric artery.

It has been found that elevation of cyclic AMP reduce agonist-mediated inositol phosphate formation in platelets (Watson *et al.*, 1984), neutrophils (Takenawa *et al.*, 1986) and A10 cultured vascular smooth muscle cells (Xuan *et al.*, 1991). It has also been reported that cyclic AMP-dependent protein kinase reduces the ability of InsP_3 to release stored Ca^{2+} through phosphorylation of the InsP_3 receptor in isolated vesicles, from brain synaptosome (Supattapone *et al.*, 1988). In the present experiments, NKH477 and dibutyryl-cyclic AMP each greatly inhibited the NA-induced increase in $[\text{Ca}^{2+}]_i$ without a corresponding change in the NA-induced synthesis of InsP_3 . Further, in β -escin-treated skinned smooth muscle, neither NKH477 nor dibutyryl-cyclic AMP modified the InsP_3 -induced Ca^{2+} release from the intracellular storage sites. These results suggest that, in smooth muscle of the rabbit mesenteric artery, NKH477 and dibutyryl-cyclic AMP inhibit the NA-induced increase in $[\text{Ca}^{2+}]_i$ without either an inhibition of the NA-induced synthesis of InsP_3 or a reduction of the sensitivity of the receptor to InsP_3 .

In smooth muscle cells, the magnitude of the increase in $[\text{Ca}^{2+}]_i$ induced by an agonist in Ca^{2+} -free solution may depend on the balance between the rates of Ca^{2+} release and Ca^{2+} removal (such as Ca^{2+} -uptake into the storage site or Ca^{2+} -extrusion to the extracellular space). When Ca^{2+} removal mechanisms are activated, the size of the increase in $[\text{Ca}^{2+}]_i$ induced by the agonist could be minimized. Conversely, the size of the agonist-induced increase in $[\text{Ca}^{2+}]_i$ would be enhanced as the rate of Ca^{2+} release induced by the agonist increases while the rate of Ca^{2+} removal remains constant. We have previously found that in Ca^{2+} -free solution, high K^+ concentration-dependently enhances the NA-induced increase in $[\text{Ca}^{2+}]_i$ without any increase in the amount of NA-induced InsP_3 synthesis (Itoh *et al.*, 1992). In the present experiments, high K^+ itself failed to increase $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution but concentration-dependently enhanced both the maximum rate of rise of $[\text{Ca}^{2+}]_i$ and the amplitude of the increase in $[\text{Ca}^{2+}]_i$. The relationship between the maximum values for the increase in $[\text{Ca}^{2+}]_i$ and the rate of rise of $[\text{Ca}^{2+}]_i$ induced by NA was linear in Ca^{2+} -free solution in the presence of various concentrations of K^+ (5.9–128 mM). Although at present the mechanism of the high K^+ -induced enhancement of the NA-induced Ca^{2+} mobilization in Ca^{2+} -free solution remains unclear, these results suggest that the magnitude of the $[\text{Ca}^{2+}]_i$ mobilization induced by NA increases in proportion to the maximum rate of rise of $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution. NKH477 and dibutyryl-cyclic AMP each greatly attenuated both the maximum rate of rise of $[\text{Ca}^{2+}]_i$ and the increase in $[\text{Ca}^{2+}]_i$ induced by NA in Ca^{2+} -free solution containing 5.9 mM K^+ . These inhibitory actions of NKH477 and dibutyryl-cyclic AP were attenuated by high K^+ , in a concentration-dependent manner and this attenuation is not due simply to the membrane depolarization induced by high K^+ because NKH477 inhibited the NA-induced increase in $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution containing 128 mM K^+ when the amount of stored Ca^{2+} was set at a low value (Figure 4). These results indicate that the extent of the inhibition induced by NKH477 or dibutyryl-

cyclic AMP on the NA-induced increase in $[\text{Ca}^{2+}]_i$ is inversely related to the rate of Ca^{2+} release induced by NA.

Caffeine (5 mM) transiently increased $[\text{Ca}^{2+}]_i$ in Ca^{2+} -free solution containing 5.9 mM K^+ . The maximum rate of rise of $[\text{Ca}^{2+}]_i$ in the presence of 5 mM caffeine was almost five times larger than that for 10 μM NA in Ca^{2+} -free solution containing 5.9 mM K^+ , but similar to that for 10 μM NA in Ca^{2+} -free solution containing 128 mM K^+ . NKH477 had no effect on the caffeine-induced increase in $[\text{Ca}^{2+}]_i$. This lack of effect might be due to the greater rate of rise of $[\text{Ca}^{2+}]_i$ in the presence of caffeine than with NA in Ca^{2+} -free solution containing 5.9 mM K^+ .

In saponin-treated skinned smooth muscle of the guinea-pig and rabbit mesenteric arteries, cyclic AMP increases the rate of Ca^{2+} re-uptake into its intracellular storage sites, as estimated from mechanical responses (Itoh *et al.*, 1982; 1985; Saida & van Breemen, 1984). In the present experiments, we found that dibutyryl-cyclic AMP increased the rate of Ca^{2+} uptake into the NA-sensitive storage sites (Figure 5). We also found that when 10 μM NA was applied twice for 30 s with a 10 min interval in Ca^{2+} -free solution, the second application of NA could still increase $[\text{Ca}^{2+}]_i$. CPA, an inhibitor of the Ca^{2+} -pump in the SR of skeletal, cardiac and smooth muscle cells (Seidler *et al.*, 1989; Kurebayashi & Ogawa, 1991; Uyama *et al.*, 1992), did not modify the increase in $[\text{Ca}^{2+}]_i$ induced by the first application of NA in Ca^{2+} -free solution, but abolished the increase induced by the second. With an application of dibutyryl-cyclic AMP during the first application of NA, the increase in $[\text{Ca}^{2+}]_i$ induced by this first application of NA was inhibited but the increase induced by the second was enhanced. CPA attenuated the inhibitory action of dibutyryl-cyclic AMP on the increase in $[\text{Ca}^{2+}]_i$ induced by the first application of NA. These results suggest that agents which increase cyclic AMP may inhibit the NA-induced increase in $[\text{Ca}^{2+}]_i$ by an activation of Ca^{2+} uptake into the storage sites.

Ca^{2+} -extrusion mechanisms (such as Na^+ - Ca^{2+} exchange or the Ca^{2+} -pump on the plasma membrane) may be activated by cyclic AMP, as reported for toad stomach smooth muscle cells (Scheid *et al.*, 1979). However, since NKH477 (0.01 μM) inhibited the increases in $[\text{Ca}^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+} -free, Na^+ -deficient solution, the Na^+ - Ca^{2+} exchange mechanism may not play an essential role in the inhibitory action of NKH477 on the NA-induced increase in $[\text{Ca}^{2+}]_i$.

In conclusion, NKH477 and dibutyryl-cyclic AMP each attenuate NA-induced Ca^{2+} mobilization with no change in either the NA-induced InsP_3 synthesis or the sensitivity of the receptor to InsP_3 . Dibutyryl-cyclic AMP increases the rate of Ca^{2+} -uptake into storage sites. Thus, it can be concluded that, in smooth muscle of the rabbit mesenteric artery, agents that increase cyclic AMP attenuate the NA-induced increase in $[\text{Ca}^{2+}]_i$ possibly through an activation of Ca^{2+} uptake into its storage sites.

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