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Nicotinamide-inhibited vasoconstriction: lack of dependence on agonist signalling pathways

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Abstract

Previously, we have shown that nicotinamide inhibits both high $[K^+]$- and phenylephrine-induced constrictions in a dose-dependent manner in rat tail arteries. We have now investigated the effect of nicotinamide on intracellular signalling pathways in vascular smooth muscle. Nicotinamide (8.2 mM) reduced the response to phenylephrine- and [Arg^8]vasopressin-induced constrictions by means of 72.9 ± 6.9 and 51.8 ± 5.7%, respectively. It also blocked phenylephrine-induced constrictions in the absence of a functional endothelium ($P < 0.0136$). In addition, pre-treatment of the artery with nifedipine (10 mM) also failed to inhibit nicotinamide's activity ($P < 0.0178$). Moreover, nicotinamide significantly reduced the sensitivity to phenylephrine in Ca^{2+}-free Krebs' solution ($P < 0.0152$). Continuous perfusion of maximal concentrations of ryanodine or thapsigargin significantly inhibited the response to phenylephrine; the addition of nicotinamide (8.2 mM) caused a significant additional inhibition when compared to the effect of ryanodine ($P < 0.0006$) or thapsigargin ($P < 0.037$) alone. In addition, β-escin (0.02%) permeabilisation and Ca^{2+} (2.5 mM)-mediated constriction was also significantly attenuated by nicotinamide ($P < 0.0001$). However, phorbol ester-induced constriction was not attenuated by nicotinamide. This would suggest that nicotinamide directly inhibits vascular smooth muscle cell contraction and is unlikely to act via blockage of external Ca^{2+} entry or release of Ca^{2+} from intracellular stores.

Keywords: Nicotinamide; Vascular smooth muscle; Cyclic ADP–ribose; Thapsigargin; Receptor-dependent Ca^{2+} channel; Ryanodine

1. Introduction

Nicotinamide has been shown to be an effective radiosensitizer of tumours in numerous animal studies (Rojas, 1992) and is currently undergoing clinical trials within the European Organization for Research and Treatment of Cancer. It has been found to increase tumour oxygenation and reduce microregional heterogeneity of blood distribution in vivo (Horsman et al., 1988; Chaplin et al., 1990). The exact pharmacological mechanism by which it increases blood flow is unclear. In a previous ex vivo study, we have shown that nicotinamide may achieve this by acting as a potent inhibitor of constriction in vascular smooth muscle. It reduced both phenylephrine and high $[K^+]$-induced constrictions in rat tail arteries in a dose dependent manner with 1 to 10 mM nicotinamide giving significant attenuation. This dose range is comparable to 1000 mg/kg commonly used to achieve effective radiosensitization in animal studies (Rojas, 1992; Horsman, 1995) as 8.2 mM (1 g/l) nicotinamide would be equivalent to 1000 mg/kg in vivo. Nicotinamide (8.2 mM) inhibited phenylephrine or high $[K^+]$-induced constrictions in rat tail artery in ex vivo perfusion experiments by up to 72% (Hirst et al., 1994). In addition, we found that nicotinamide is also active in rat tumour arteries where it significantly reduces the spontaneous rhythmic constrictions observed in these vessels (Kennovin et al., 1994; Hirst et al., 1995). Nicotinamide's action in the rat tail arteries was not blocked by *N*ω-nitro-L-arginine methyl ester (L-NAME), indomethacin or propranolol indicating that it does not act through endothelial stimulation of nitric oxide, prostaglandins or β-adrenergic stimulation of cAMP (Hirst et al., 1994). Therefore, given our previous findings, it is possi-

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ble that nicotinamide acts either via other endothelial-derived relaxing factors or, more likely, at a post-receptor level within the smooth muscle cells.

To date a number of intracellular pathways controlling constriction have been identified in vascular smooth muscle. External Ca^{2+} enters through high $[K^+]$-stimulated opening of voltage-dependent Ca^{2+} channels or phenylephrine-induced opening of receptor-dependent Ca^{2+} channels. The increase in intracellular Ca^{2+} causes a cascade leading to constriction (Van Breemen and Saida, 1989). Membrane bound phospholipase C is also activated by phenylephrine or vasopressin in smooth muscle, generating two transient second messengers, inositol trisphosphate (IP3) and diacylglycerol (Doyle and Ruegg, 1985; Bylund, 1988). IP3 mobilises Ca^{2+} from the sacroplasmic reticulum while diacylglycerol activates protein kinase C (Somlyo and Himpens, 1989; Berridge, 1993). More recently, another mechanism of intracellular Ca^{2+} release has been described in sea urchin eggs, termed Ca^{2+}-induced Ca^{2+} release (Clapper et al., 1987). This is mediated by the second messenger cyclic ADP–ribose which stimulates the opening of the ryanodine receptor (Lee et al., 1989). Cyclic ADP–ribose may play a role in vascular smooth muscle constriction, as Ca^{2+}-induced Ca^{2+} release via opening of the ryanodine receptor, has been reported in various smooth muscle systems (Wagner-Mann et al., 1992; Kuemmerle et al., 1994; Lynn and Gillespie, 1995). Much less is known about this pathway in vascular smooth muscle. However, phenylephrine is thought to induce cyclic ADP–ribose production, because the addition of ryanodine attenuated phenylephrine-induced constriction in rat aorta and dog mesenteric artery (Low et al., 1993).

The lack of understanding of the exact mechanism of action of nicotinamide has contributed to the failure of efforts to produce analogs with improved efficacy (Horsman et al., 1986; Brown et al., 1991). Using the rat tail artery perfusion system as a model for studying the mechanism of action of nicotinamide as a radiosensitizer, we have investigated the role played by the endothelium in nicotinamide-mediated activity and the effect of nicotinamide on various known intracellular pathways which lead to constriction in vascular smooth muscle.

2. Methods

2.1. Apparatus

The apparatus used to perfuse rat tail arteries has been described in detail previously (Hirst et al., 1994). Male adult normotensive Wistar rats were killed by asphyxiation and cervical dislocation and a 1 cm section of artery was cannulated and dissected from the tail. Four artery sections were simultaneously perfused internally and externally with oxygenated (95% O_2, 5% CO_2) Krebs' solution (in mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1 and glucose 5.6) in an organ bath maintained at 35°C. The back pressure measured using differential pressure transducers (RS Components, 0–5 psi) is proportional to the degree of constriction in the artery. The internal flow rate was increased incrementally over a 30 min period to 2 ml/min. Arteries were then left for a further 30 min to allow stabilization of the arterial tone before drugs were added to the preparation.

2.2. Perfusion experiments

Transient constrictions were induced with bolus injections (10 ml) of phenylephrine (10^{-6} to 10^{-3} M) or $[Arg^8]$vasopressin (vasopressin) (10^{-6} to 10^{-5} M). Nicotinamide (8.2 mM) nifedipine (10 mM) or ryanodine (1 mM) were perfused continuously in the external and internal perfusate for 30 min and 1 h, respectively, before the vasoconstrictor bolus cycle was repeated. Peak perfusion pressure measurements were used to construct log dose–response curves for phenylephrine or vasopressin alone and in the presence of nicotinamide. Sustained constrictions were also induced by continuously perfusing phorbol esters (1 mM) or phenylephrine (10 mM) in the internal and external perfusate.

2.2.1. Endothelium-denuded arteries

After performing phenylephrine bolus cycles in the presence and absence of nicotinamide, the endothelium was removed by pumping air through the artery sections for 10 s. Removal was verified by the loss of the inhibitory effect of 10 mM acetylcholine on an elevated vasoconstrictor tone elicited by the continuous perfusion of 10 mM phenylephrine (data not shown). The phenylephrine bolus cycle was carried out while two arteries were used as the control and simultaneously two were treated with nicotinamide.

2.2.2. Calcium-free experiments

Artery sections were initially perfused in normal Ca^{2+} Krebs' solution until the 30 min stabilization period was complete. Internal and external perfusates were then changed to Ca^{2+}-free Krebs' solution containing 50 mM EDTA. After a further 30 min stabilization period, a phenylephrine bolus cycle was carried out in the absence and then in the presence of nicotinamide.

2.2.3. Thapsigargin and ryanodine experiments

Initially, experiments were carried out to determine maximal effective doses of ryanodine and thapsigargin in the rat tail artery smooth muscle. Phenylephrine (10 mM) was perfused continuously in the presence of thapsigargin (0.1 or 1 mM) for 30 min followed by 15 min in normal Krebs' solution to remove phenylephrine and thapsigargin. As thapsigargin is an irreversible inhibitor further exposure was unnecessary to maintain its effect on the Ca^{2+} status of the sarcoplasmic reticulum (Low et al., 1993). The

arteries were then exposed to Ca^{2+}-free Krebs' solution for a further 15 min to remove Ca^{2+} from the extracellular space. Application of phenylephrine (10 mM) in Ca^{2+}-free Krebs' solution was performed to test for Ca^{2+} release from sarcoplasmic reticulum. After pre-treatment with 0.1 mM thapsigargin, a transient constriction was recorded upon perfusion of phenylephrine in Ca^{2+}-free Krebs' solution. This constriction had a peak pressure of 22.5 mm Hg $\pm$3.63 and decreased rapidly with a mean duration of 7.7 min $\pm$1.7 ($n = 6$). When the concentration of thapsigargin was raised to 1 mM, this transient constriction was absent, indicating that the sarcoplasmic reticulum did not contain Ca^{2+}. To determine the maximal dose of ryanodine, up to 1 mM ryanodine was perfused for 1 h prior to treatment with Ca^{2+}-free Krebs' followed by phenylephrine (10 mM) in Ca^{2+}-free Krebs' solution as above. Following treatment with 1 mM ryanodine, no constriction was recorded in the presence of phenylephrine. Thus, for all experiments testing the effect of nicotinamide after sarcoplasmic reticulum depletion, 1 mM ryanodine or 1 mM thapsigargin was used. All experiments testing the effect of nicotinamide on sarcoplasmic reticulum refilling were executed as follows: phenylephrine (10 mM) was perfused continuously for 30 min in the presence of thapsigargin (1 mM). Initially, an elevated vasoconstrictor tone was obtained, but this decreased to new a plateau indicating loss of functional sarcoplasmic reticulum. Krebs' solution alone was perfused for 30 min before phenylephrine bolus cycles were performed in the absence and presence of nicotinamide.

2.2.4. Experiments using permeabilised arteries

β-escin (0.02%) was perfused in Ca^{2+}-free Krebs' for 30 min in the presence of calmodulin (10 nM). The permeabilised arteries were then perfused with Ca^{2+} containing Krebs' $\pm$ nicotinamide (8.2 mM) in the presence of β-escin.

2.3. Chemicals and reagents

Nicotinamide, phenylephrine, vasopressin, thapsigargin, ryanodine, nifedipine, phorbol 12-myristate 13-acetate (PMA) and acetylcholine were obtained from Sigma. Thapsigargin and PMA was dissolved in dimethyl sulphoxide as 1 mM stock solution and diluted to 1 mM in Krebs' solution on the day of use. Phenylephrine, nicotinamide, vasopressin, ryanodine and acetylcholine were dissolved in Krebs' solution ($\pm Ca^{2+}$) on the day of use.

2.4. Statistical analysis

Each result was confirmed in at least three independent experiments. Analysis of variance (ANOVA) was carried out using Statview for Macintosh with 95% confidence limits with factorial analysis. The Student's *t*-test was used to test for significance in the experiments using phorbol esters and skinned arteries.

3. Results

3.1. The effect of nicotinamide in endothelium-denuded arteries

Nicotinamide could be achieving vasorelaxation of smooth muscle wholly or in part by stimulating vasorelaxant release from the endothelium. We investigated this possibility by examining the effect of nicotinamide on phenylephrine-induced vasoconstriction in arteries without functional endothelium. Dose–response curves to phenylephrine bolus injections (5×10^{-6} to 1×10^{-3} M) were constructed for these vessels (Fig. 1). In the presence of nicotinamide (8.2 mM), the response to phenylephrine concentrations from 1×10^{-5} up to 1×10^{-3} M was significantly reduced ($P < 0.0136$) with a mean reduction of 78 $\pm$ 5.1%

3.2. The effect of nicotinamide on extracellular calcium entry

To identify if nicotinamide prevented entry of extracellular Ca^{2+}, we designed experiments that examined the effect of nicotinamide on arterial constriction generated in the absence of external Ca^{2+} or in the presence of the Ca^{2+} channel blocker nifedipine. In Krebs' solution containing $CaCl_2$ (2.5 mM), nicotinamide (8.2 mM) significantly attenuated phenylephrine-induced transient vasoconstrictions by 72.92 $\pm$ 6.9% ($P < 0.0196$) at all phenylephrine concentrations tested. In Ca^{2+}-free Krebs' solution containing EDTA (50 mM), the response to phenylephrine was lower than that in Ca^{2+}-containing Krebs' solution,

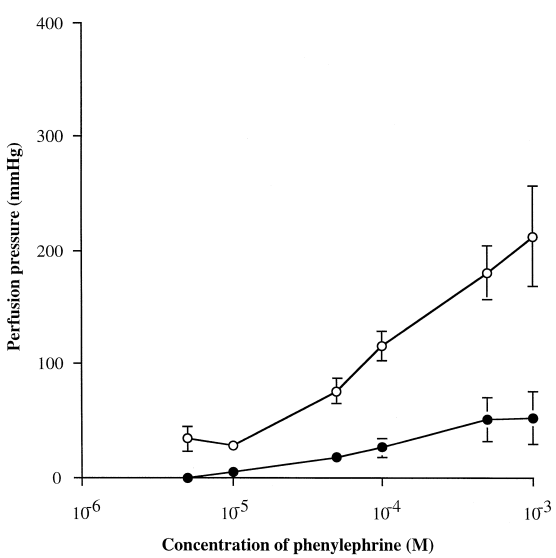

Fig. 1. Dose response to phenylephrine in endothelial denuded rat tail arteries in Krebs' only (—○—) and in the presence of nicotinamide (8.2 mM) (—●—). Data represent the means $\pm$ S.E. mean for four arteries. Responses to phenylephrine above 1×10^{-5} M in the presence of nicotinamide are significantly reduced compared to those in Krebs' only ($P \leq 0.0136$).

but nicotinamide significantly reduced this constriction by a mean of 68.12 ± 11.47% ($P < 0.0152$) (Fig. 2). In Krebs' solution containing Ca^{2+} (2.5 mM) and nifedipine (10 mM), nicotinamide significantly inhibited the response to bolus injections of phenylephrine ($P < 0.0178$) (Fig. 3).

3.3. The effect of nicotinamide on vasopressin-induced constriction

Bolus injections of vasopressin from 10^{-6} to 10^{-5} M were used to generate a dose–response curve. In the presence of nicotinamide (8.2 mM), this response was reduced by a mean of 51.77 ± 5.67%, with vasopressin doses above 2×10^{-6} showing significant differences ($P < 0.0371$) (Fig. 4).

3.4. The effect of ryanodine on nicotinamide-mediated inhibition of constriction

Ryanodine binds to the cyclic ADP–ribose Ca^{2+} release channel on the sarcoplasmic reticulum locking it in the open position, thus preventing Ca^{2+}-induced Ca^{2+} release. Pre-incubation of the rat tail arteries for 1 h with ryanodine (1 mM) produced a transient increase in constriction but previous basal tone was attained before the end of this pre-incubation. The phenylephrine log dose response ($P < 0.0034$) was significantly reduced indicating that generation of cyclic ADP–ribose and subsequent Ca^{2+} release from the sarcoplasmic reticulum may be a major contributor to phenylephrine-induced contraction in this model (Fig. 5). The degree of inhibition by ryanodine was similar to that achieved by nicotinamide at the concentration used here. If nicotinamide blocks production of cyclic ADP–ribose, then it might be expected to have no further effect in the presence of ryanodine. However, we

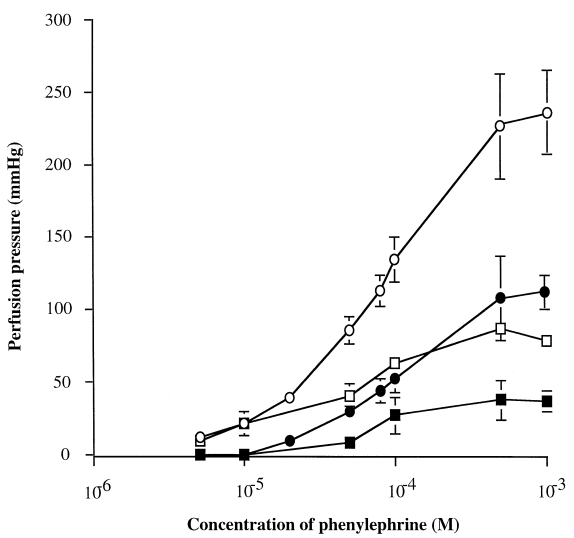

Fig. 2. Dose response to phenylephrine in Krebs' with normal Ca^{2+} (2.5 mM) (—○—), in nicotinamide (8.2 and 2.5 mM Ca^{2+}) (—●—), in Ca^{2+}-free Krebs' with EDTA (50 μM) (—□—) and in Ca^{2+}-free Krebs' with EDTA (50 μM) and nicotinamide (8.2 mM) (—■—). In Ca^{2+}-containing Krebs', responses to phenylephrine doses between 5×10^{-6} and 5×10^{-4} M were significantly reduced in the presence of nicotinamide ($P \leq 0.0196$). Data represent the means ± S.E. mean for four arteries. In Ca^{2+}-free Krebs' with EDTA, responses to phenylephrine concentrations from 1×10^{-5} to 1×10^{-3} were significantly reduced in nicotinamide ($P \leq 0.0152$). Data represent the means ± S.E. for three arteries.

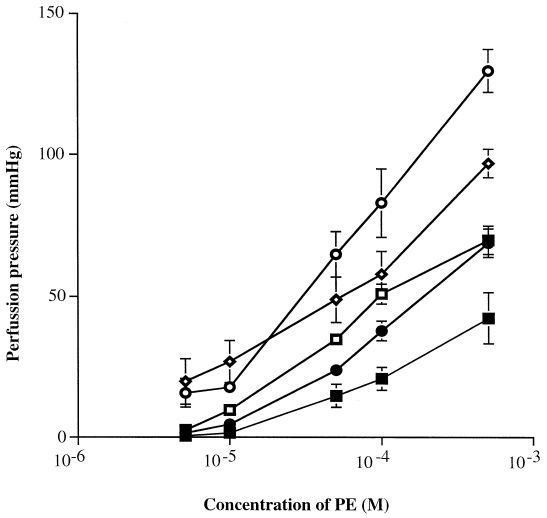

Fig. 3. Dose response to phenylephrine in Kreb's only (—○—) nicotinamide (8.2 mM) (—●—) nifedipine (10 μM) (—□—), nicotinamide in combination with nifedipine (10 μM) (—■—) and Krebs' washout (—◇—). Data represent the means ± S.E. mean for three arteries. All responses to phenylphrine in the presence of nifedipine are significantly different from those in Krebs' only ($P < 0.0178$). Responses to phenylephrine in nicotinamide and nifedipine in combination are significantly different from those in nipedipine or nicotinamide alone ($P < 0.005$).

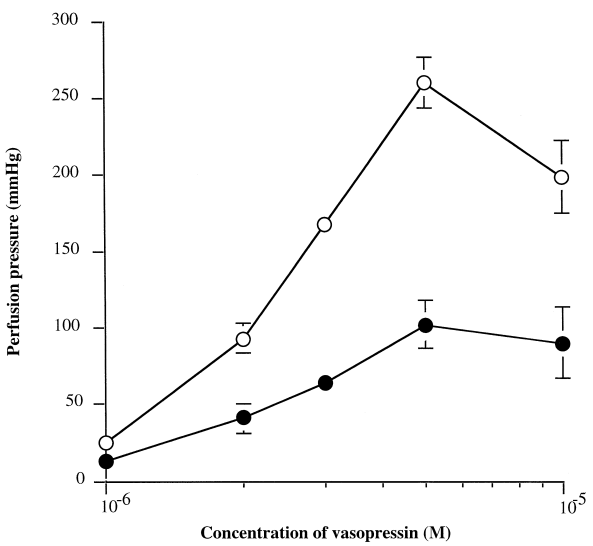

Fig. 4. Dose response to vasopressin in Krebs' only (—○—) and in nicotinamide (8.2 mM) (—●—). In the presence of nicotinamide, responses to vasopressin between 2×10^{-6} and 1×10^{-5} M were significantly reduced ($P \leq 0.037$). Data represent means ± S.E. for six arteries.

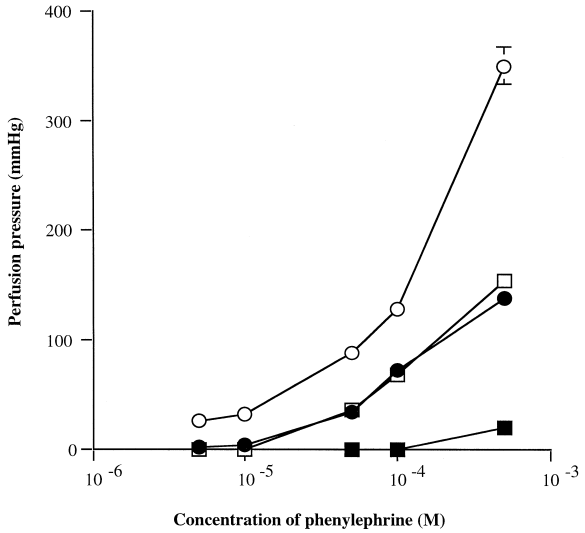

Fig. 5. Dose response to phenylephrine in Krebs' only (—○—) nicotinamide (8.2 mM) (—●—) ryanodine (1 μM) (—□—) and nicotinamide (8.2 mM) in combination with ryanodine (1 μM) (—■—). Data represent the means ± S.E. mean for three arteries. All responses to phenylephrine in the presence of ryanodine are significantly different from those in Krebs' only ($P \leq 0.0034$) and responses to phenylephrine (from 50 μM to 50 mM) in nicotinamide and ryanodine in combination are significantly different from those in ryanodine or nicotinamide alone ($P \leq 0.0006$).

found that in combination, nicotinamide and ryanodine were markedly additive, producing a profound reduction in the dose response to phenylephrine over the range 5×10^{-5} to 5×10^{-4} compared to that obtained with either nicotinamide or ryanodine alone ($P < 0.0006$) (Fig. 5).

3.5. The effect of thapsigargin on nicotinamide-mediated inhibition of constriction

It is possible that nicotinamide affects vasodilation by increasing repletion of the sarcoplasmic reticulum or by slowing Ca^{2+} release from its stores. We have carried out experiments to establish if nicotinamide can cause vasodilation in vascular smooth muscle without a functional sarcoplasmic reticulum. Continuous perfusion of phenylephrine (10 mM) was used to stimulate Ca^{2+} release from the sarcoplasmic reticulum; the presence of thapsigargin (1 mM), a selective irreversible inhibitor of a Ca^{2+}-ATPase pump on the sarcoplasmic reticulum prevented repletion. Initially, this gave an elevated tone which decreased to 44% of this value. Following washout with Krebs' solution, a dose response to phenylephrine bolus injections was then generated in the presence and absence of nicotinamide (8.2 mM) (Fig. 6). Inhibition of the sarcoplasmic reticulum Ca^{2+} pump with thapsigargin reduced the dose response to phenylephrine. In the presence of nicotinamide, phenylephrine doses below 10^{-4} M no longer gave a measurable response and constrictions at 5×10^{-4} and 1×10^{-3} M phenylephrine were significantly reduced ($P < 0.037$).

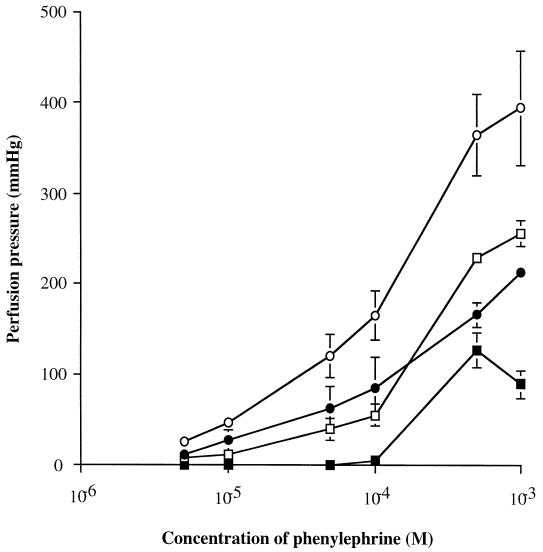

Fig. 6. Dose response to phenylephrine in Krebs' only (—○—) nicotinamide (8.2 mM) (—●—) thapsigargin (1 μM) (—□—) and nicotinamide (8.2 mM) in combination with thapsigargin (1 μM) (—■—). Data represent the means ± S.E. mean for three arteries. After treatment with thapsigargin, all responses to phenylephrine were significantly reduced by nicotinamide ($P \leq 0.037$).

3.6. The effect of nicotinamide on phorbol ester-induced contractions

A sustained constriction was induced by perfusing PMA (1 mM) through the internal and external perfusate. Nicotinamide had no significant effect on the phorbol-induced constriction (Fig. 7).

3.7. The effect of nicotinamide on Ca^{2+}-induced constrictions in β-escin permeabilized arteries

The addition of Ca^{2+} in the presence of β-escin induced a substantial contraction. The presence of nicotin-

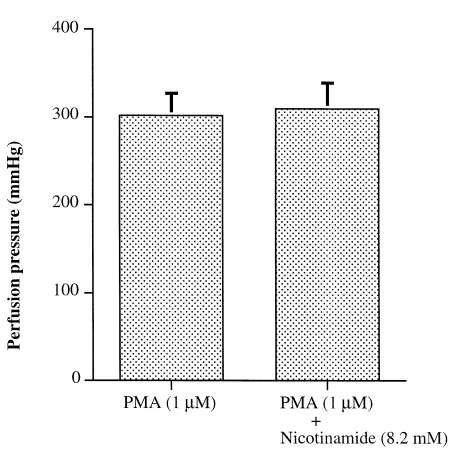

Fig. 7. The effect of nicotinamide (8.2 mM) on the continuous perfusion of PMA (1 μM) in rat tail arteries. Data represent the means ± S.E. mean for four arteries.

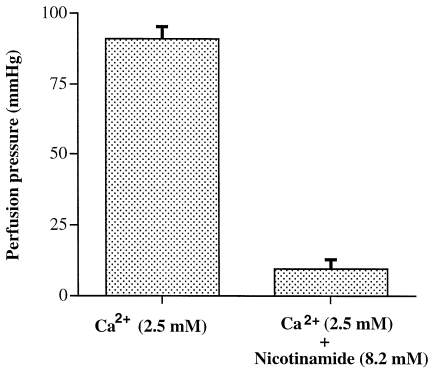

Fig. 8. The effect of nicotinamide (8.2 mM) on Ca^{2+}-induced constrictions in arteries permeabilised with β-escein (0.02%). Data represent means ± S.E. mean for three arteries. Nicotinamide reduced the Ca^{2+}-mediated constriction by 89.4% ($P < 0.0001$).

amide significantly attenuated this response ($P < 0.0001$) (Fig. 8).

4. Discussion

It is well documented that the vascular endothelium can promote vasodilation via secretion of endothelium-derived relaxing factor (EDRF) and smooth muscle hyperpolarisation factors (Furchgott and Vanhoutte, 1989; Brayden, 1990; Chen et al., 1991). Vasodilators such as bradykinin and acetylcholine exert their action by inducing endothelial secretion of nitric oxide in some blood vessels (Palmer et al., 1987; Hwa et al., 1994). Our previous data showed that nicotinamide did not stimulate endothelial release of nitric oxide, prostaglandins or β-adrenoceptor activation as the presence of L-NAME, indomethacin or propanolol did not block nicotinamide-mediated vasorelaxation (Hirst et al., 1994). However, the stimulation by nicotinamide of other endothelium-derived factors was still a possibility as both bradykinin and acetylcholine have been shown to cause vasodilation by a mechanism which is independent of nitric oxide (Beny and Brunet, 1988; Kauser and Rubanyi, 1992). Our data obtained with endothelium-denuded artery sections demonstrate that the endothelium does not play a significant role in nicotinamide-mediated inhibition of constriction. However, given that the effector of acetylcholine-induced relaxation can vary depending on the artery studied (Hwa et al., 1994), it may be possible that the endothelium of other arteries such as tumour vessels would respond differently to nicotinamide.

Data presented here show that nicotinamide also significantly blocked vasopressin-induced contractions in the rat tail artery in a similar manner to those induced by phenylephrine or high $[K^+]$. Since vasopressin is reported to generate IP3 and diacylglycerol in smooth muscle cells it appears that nicotinamide may also block smooth muscle contraction initiated by activation of phospholipase C. It has been reported that vasopressin also induces Ca^{2+} influx in cultured smooth muscle cell lines (Wu et al., 1995). This raised the possibility that nicotinamide acts by blocking Ca^{2+} channel opening, a process which would be common to vasopressin, phenylephrine and high $[K^+]$-induced constrictions. This prompted the experiments under Ca^{2+}-free conditions and using nifedipine. In the absence of extracellular Ca^{2+}, phenylephrine-induced constrictions were reduced as expected, due to the absence of external Ca^{2+} influx upon opening of receptor dependent channels. The ED50 of the dose response to phenylephrine in the presence of Ca^{2+}-containing Kreb's solution was 1.2×10^{-4} M while it was 2.5×10^{-4} M in Ca^{2+}-free Krebs' solution. This indicates that Ca^{2+} mobilisation from internal stores accounts for the generation of a substantial proportion of the contraction in this preparation. Significantly, nicotinamide markedly reduced the responses to phenylephrine in Ca^{2+}-free Krebs' solution and in the presence of nifedipine. Thus, nicotinamide's inhibitory activity is unlikely to be based on any interaction with plasmalemmal Ca^{2+} channels.

Cyclic ADP–ribose production is initiated by activation of the membrane bound protein CD38. This marker contains a bifunctional enzyme whose activity is responsible for the production and inactivation of cyclic ADP–ribose (Rusinko and Lee, 1989; Howard et al., 1993). The enzyme conversion of ADP–ribose to cyclic ADP–ribose utilises β-NAD^+ and produces nicotinamide as a by-product. In other cells such as Aplysia ovatestis where the ADP–ribose cyclase enzyme shows a high degree of homology with the mammalian CD38 antigen, the presence of nicotinamide decreased the activity of the cyclase enzyme and cyclic ADP–ribose formation (Inageda et al., 1995). Half maximal inhibition was observed at 1 mM nicotinamide, giving rise to the possibility that nicotinamide might block the formation of cyclic ADP–ribose and the subsequent intracellular Ca^{2+} release in the rat tail artery smooth muscle cells. In our experiments, we continuously perfused ryanodine which has been shown to lock the cyclic ADP–ribose receptor in a sub-conductance state when used at micromolar concentrations (Meissner, 1986). This ensured that the cyclic ADP–ribose releasable Ca^{2+} store was empty. The significant reduction in the phenylephrine dose response in the presence of ryanodine underlines the possible importance of cyclic ADP–ribose in vascular smooth muscle contraction despite reports that the expression of the receptor in smooth muscle is weak (McPherson and Campbell, 1993; Sorrentino and Volpe, 1993). This would also corroborate the findings from our experiments with Ca^{2+}-free Krebs' solution that there is a substantial intracellular Ca^{2+} store in our artery system. While it is well established that ryanodine, or its endogenous messenger, have no affinity for the IP3 receptor, there is considerable evidence to suggest that these Ca^{2+} stores overlap in smooth muscle (Dargie et al., 1990). Our initial experiments with ryanodine would support this as there was no response to phenylephrine in Ca^{2+}-free Krebs'

solution following ryanodine pretreatment. Thus, ryanodine may also be emptying Ca^{2+} stores which could be released by binding of IP3 to its receptor. Nicotinamide further reduced the phenylephrine dose response in combination with ryanodine indicating that nicotinamide-induced inhibition of constriction does not depend on inhibition of CD38 ribosyl cyclase activity.

The study of Ca^{2+} release and the role played by the sarcoplasmic reticulum in smooth muscle contraction has been greatly facilitated by the use of the selective Ca^{2+}-ATPase pump inhibitor, thapsigargin (Ristiensen and Hanley, 1994; Thastrup et al., 1994). Furthermore, it has been demonstrated that application of thapsigargin and phenylephrine in combination depleted the phenylephrine releasable intracellular Ca^{2+} store and prevented its refilling in rat aorta (Low et al., 1993). Our results show that phenylephrine continues to evoke contractions at a reduced amplitude in the absence of a functional sarcoplasmic reticulum and that nicotinamide remains a potent inhibitor of phenylephrine-induced constriction after thapsigargin treatment under these circumstances. This demonstrates that nicotinamide does not achieve inhibition of constriction in rat tail artery by enhancing sarcoplasmic reticulum repletion or blocking any second messengers which release intracellular Ca^{2+}.

Phorbol esters are well recognised specific activators of protein kinase C and they have been shown to induce slow sustained contractions in smooth muscle (Castagna et al., 1982). Protein kinase C is believed to directly activate the contractile process by phosphorylating myosin light chains. Thus, phorbol esters provide a useful tool for the study of the contractile mechanism of smooth muscle without activating intracellular signalling. The fact that nicotinamide does not have any effect on these contractions strongly suggests that nicotinamide acts independently of actin–myosin coupling. Furthermore, the inhibitory effect of nicotinamide on the Ca^{2+}-induced constrictions in the permeabilised arteries could suggest that nicotinamide is somehow acting on the Ca^{2+} activation of calmodulin and myosin light chain kinase. The common molecules for all of the other signalling pathways (phenylephrine, vasopressin and high $[K^+]$) are calmodulin and myosin light chain kinase. All of the results presented in this paper agree with the assertion that nicotinamide could attenuate their role in contraction.

In conclusion, our results demonstrate that nicotinamide blocks vasoconstriction in rat tail artery in an endothelium-independent manner. It achieves this via a mechanism that does not appear to target specific parts of intracellular signalling pathways known to cause contraction in smooth muscle cells. Given the lack of dependence on the contractile agonist used it is possible that nicotinamide achieves its effects by acting close to the myosin or actin proteins which are common to all pathways. However, the experiments with the phorbol ester and permeabilised arteries strongly suggest that nicotinamide does not act on the actin–myosin coupling, but it may alter the activation of Ca^{2+} calmodulin or myosin light chain kinase. Alternatively, it could enhance the adenylate or guanylate cyclase mediated relaxation. Nevertheless, we cannot rule out the possibility that nicotinamide may have a number of sites of action within vascular smooth muscle control. These possibilities are currently under investigation.

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