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Mechanism of butyrate-induced vasorelaxation of rat mesenteric resistance artery

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1 The vasorelaxant effect of the sodium salt of the short chain fatty acid, butyrate, on preconstricted rat small mesenteric arteries (mean inner diameter approximately 300 μm) was characterized. Isometric force development was measured with a myograph, and intracellular pH (pH_i) was simultaneously monitored, in arteries loaded with the fluorescent dye BCECF in its acetomethoxy form. Sodium butyrate (substituted isosmotically for NaCl) was applied to arteries after noradrenaline (NA) or high K^+ contractures were established.

2 Arteries preconstricted with a concentration of NA inducing an approximately half maximal contraction were relaxed by 91.5±6.3% by 50 mmol l^{-1} butyrate. This concentration of butyrate did not, however, cause a significant relaxation of contractures to a maximal (5 μmol l^{-1}) NA concentration, and also failed to relax signifcantly contractures stimulated by high (45 and 90 mmol l^{-1}) K^+ solutions. Contractures elicited with a combination of NA (at a submaximal concentration) and 45 mmol l^{-1} K^+ were, however, markedly relaxed by butyrate.

3 Investigation of the concentration-dependency of the butyrate-induced relaxation of the half maximal NA response revealed an EC_{50} for butyrate of approximately 22 mmol l^{-1}.

4 Sodium butyrate (50 mmol l^{-1}) caused pH_i to decrease from 7.25±0.02 to 6.89±0.08 ($n=4$, $P<0.001$). However, the vasorelaxant effect of butyrate on the submaximal NA contracture was not significantly modified when this fall in intracellular pH was prevented by the simultaneous application of NH_4Cl.

5 Butyrate-induced relaxation was also unaffected by endothelial denudation and inhibition of NO synthase with N^{ω}-nitro-L-arginine methyl ester (100 μmol l^{-1}).

6 The relaxation of the NA contracture by 50 mmol l^{-1} sodium butyrate was abolished in arteries pretreated with the cyclic AMP antagonist $\mathbf{R_p}$-cAMPS (25 μmol l^{-1}).

7 We conclude that the butyrate-induced relaxation of the NA contracture is independent of intracellular acidification. The ability of $\mathbf{R_p}$-cAMPS to abolish the butyrate relaxation indicates that stimulation of the cyclic AMP second messenger system may play an important role in mediating this effect.

Keywords: Rat mesenteric arteries; vasorelaxation; intracellular pH; butyrate; BCECF; cyclic AMP; $\mathbf{R_p}$-cAMPS; endothelium

Introduction

Exposure of blood vessels to short chain fatty acids (SCFA) such as lactate, acetate, and butyrate is known to induce vasodilatation (Mortensen *et al.*, 1990; Nutting *et al.*, 1991; Austin & Wray, 1993; 1994). The mechanism underlying SCFA-mediated relaxation remains unclear. Evidence that a reduction in intracellular pH causes relaxation was obtained by Okada *et al.* (1991) who observed that intracellular acidification with a proton ionophore diminished the vasopressin-induced contraction, and Austin & Wray (1993; 1994), who found that 40 mmol l^{-1} Na butyrate caused a relaxation of high K^+ depolarized rat mesenteric resistance arteries which was associated with a fall in pH_i. On the other hand, a number of studies have established that the acute reduction of pH_i results in a rise in tension in vascular smooth muscle cells (Spurway & Wray, 1986; Matthews *et al.*, 1992; Jensen *et al.*, 1993; Iino *et al.*, 1994).

It has also been observed that acetate, proprionate, and butyrate cause increases in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in the rat caudal artery (Daugirdas *et al.*, 1988; Nutting *et al.*, 1991), although a causal relationship between this effect and vasorelaxation was not proved. The effect of acetate was shown not to depend on the production of adenosine (Nutting *et al.*, 1992). Conversely, in calf pulmonary arteries preconstricted with high K^+ solution, and human placental arteries and veins preconstricted with $PGF_{2\alpha}$ lactate induced a relaxation which was inhibited by hypoxia and methylene blue, which among other actions blocks guanylyl cyclase. On the basis of these and other observations, Omar and co-workers (Omar *et al.*, 1993a, b) proposed that lactate was acting to increase intracellular H_2O_2, which then activated guanylyl cyclase to increase intracellular cyclic GMP.

In the present study, we have examined the mechanism by which the sodium salt of the SCFA, butyrate, causes vascular relaxation in rat isolated mesenteric resistance arteries, using an approach based on the simultaneous measurement of force development and intracellular pH. We provide evidence that the action of butyrate is independent of cellular acidification, and is prevented by the cyclic AMP antagonist, $\mathbf{R_p}$-cAMPS.

Methods

Preparation of arteries

The investigation was performed in accordance with the Home Office *Animals (Scientific Procedures) Act 1986*, published by

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Her Majesty's Stationary Office, London. Male Wistar rats, (weight 250–300 g), were killed by cervical dislocation. The mesentery was removed and resistance arteries, (approx. i.d. 300 μm), were dissected free from the surrounding tissue and mounted as isometric preparations on either a Mulvany-Halpern myograph (for the measurement of tension alone) or a computer controlled myograph (Cambustion Ltd, Cambridge, UK) for the simultaneous determination of tension and pH_i. The arteries were bathed in physiological saline solution (PSS), containing (in mmol l^{-1}): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.289, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and glucose 5.5, and gassed with 5% CO_2/95% O_2. The arteries were set to a circumference 90% of that obtained when the vessels were stretched to a transmural pressure of 13.4 KPa (Mulvany & Halpern, 1977). Prior to experiments, arteries were subjected to a routine 'run-up' procedure consisting of one contraction to K^+ substituted PSS, (equimolar substitution of KCl for NaCl), one to 5 μmol l^{-1} noradrenaline (NA) and three to 5 μmol l^{-1} NA and KCl substituted PSS.

pH_i measurement

Following mounting on the myograph, arteries were incubated for 1 h in PSS containing 5 μmol l^{-1} 2′,7′-*bis*(carboxymethyl)-5-(6′)-carboxyfluorescein acetoxy methyl ester, (BCECF-AM, Calbiochem, Nottingham). The myograph was mounted on a stage of a Nikon TMD inverted microscope with an epi-fluorescence attachment. The artery was alternately excited with light of wavelength 490 and 430 nm at 64 Hz and emitted light from the excited dye was monitored at 550 nm using a barrier filter and a photomultiplier tube, the output of which was digitized, stored, and displayed on a microcomputer using a spectrophotometer system (Cairn Ltd, Sittingbourne, Kent). The 490:430 ratio was then used to calculate the pH_i, following calibration in each artery with a medium containing 140 mmol l^{-1} potassium and 10 μmol l^{-1} nigericin (Thomas *et al.*, 1979) in which this ratio was recorded at a minimum of six pH levels (pH adjusted with 0.1 M potassium hydroxide). Linear regression of pH_i *vs* the 490:430 ratio was greater than 0.995 for all arteries.

Relaxation to butyrate

Preliminary experiments showed that butyrate-induced relaxation of the NA contracture was consistently observed when the response to NA was sub-maximal; conversely, butyrate had little effect when applied in the presence of concentrations of NA producing a maximal response (see Results). For this reason, an initial NA concentration-response analysis was performed in most experiments, and a concentration sufficient to produce approximately 50% of the maximum contractile response was selected for testing the effect of butyrate. Na butyrate was substituted for NaCl. This substitution was always isosmotic except for the slightly hyperosmotic solution containing 90 mmol l^{-1} K^+, in which 50 mmol l^{-1} Na butyrate replaced 34 mmol l^{-1} NaCl. In order to examine the effects of butyrate, arteries were stimulated with NA or high K^+ solution; 3 min after the stimulus was applied, the solution was replaced with one containing Na butyrate in addition to the stimulating agent, for a further 10 min. The effect of butyrate was then measured as the change in the amplitude of the contracture between 3 and 13 min. In order to assess the intrinsic time-dependent development of the response between 3 and 13 min in the absence of butyrate, a 'time control' response was recorded in each artery. Arteries were contracted with NA or high K^+ and after 3 min this solution was replaced by an identical one for a further 10 min.

Removal of endothelium or inhibition of NO synthase

Removal of the endothelium was achieved by passing a human hair through the lumen of the mounted vessel several times. The presence of an intact endothelium was assessed in all arteries by the demonstration of >80% relaxation to 10^{-6} M acetylcholine, (ACh), in vessels preconstricted with 1 μmol l^{-1} NA. Successful removal of the endothelium was demonstrated by <10% ACh-induced vasorelaxation. In some experiments, NO synthase was inhibited by incubating the vessel with 100 μmol l^{-1}, N^{ω}-nitro L-arginine methyl ester, (L-NAME; Sigma Chemical Co., Dorset) for 20 min. All subsequent solutions also contained L-NAME.

Chemicals and drugs

Adenosine 3′:5′-cyclic monophosphothioate, R_p isomer (R_p-cAMPS) was obtained from Calbiochem-Novabiochem, (Nottingham). Methylene blue was obtained from the Pharmacy at St. Thomas' hospital. All other chemicals were obtained from Sigma Chemical Company, Ltd. (Poole, Dorset).

Statistical analysis

The *n* values listed in the text for each experiment represent the number of animals in which that experiment was repeated. Force at the end of the 10 min exposure to butyrate was compared to that measured in the time control contractures from each artery. Data were compared by Student's paired *t* test and a value of $P<0.05$ was considered to indicate a significant effect. 'NS' in the text indicates the lack of a significant difference between two sets of data.

Results

Vasorelaxation induced by butyrate

The ability of Na butyrate to relax preconstricted mesenteric artery depended upon the nature and strength of the constricting stimulus. When the preconstriction was evoked with NA at its approximate EC_{50}, 50 mmol l^{-1} butyrate caused a rapid and almost complete relaxation (Figure 1a). Measurement of tension at the end of the 10 min exposure to butyrate revealed that 50 mmol l^{-1} butyrate produced a relaxation of 91.5±6.3% compared to the 15.4±3.4% decay of the contracture observed over the same time in the time controls ($n=21$, $P<0.001$).

The lower panel of Figure 1a illustrates the typical effect of butyrate on pH_i observed in this experiment. NA induced a small but significant fall in pH_i from 7.29±0.01 to 7.25±0.02, ($n=4$, $P<0.02$). The addition of 50 mmol l^{-1} butyrate subsequently caused a large further fall in pH_i to 6.89±0.08 ($n=4$), which was highly significant ($P<0.001$). Butyrate did not affect the pH of the bathing media.

Arteries pre-constricted with a concentration of NA causing a maximal contracture showed little or no relaxation in response to 50 mmol l^{-1} butyrate. Figure 1b illustrates a typical experiment, in which 50 mmol l^{-1} butyrate had little effect on the contracture evoked by the maximal concentration of 5 μmol l^{-1} NA, although it completely relaxed the sub-maximal contracture to 1.75 μmol l^{-1} NA. Butyrate (50 mmol l^{-1}) also caused little or no relaxation when applied during contractures induced by high K^+ depolarization. Figure 2a, for example, shows that butyrate had no observable effect upon the contracture elicited by 45 mmol l^{-1} K^+, although it again completely relaxed the half-maximal NA contracture in this artery (not shown). Experiments were also carried out using 90 mM K^+ as the preconstricting agent. When this higher concentration of K^+ was used, 50 mM butyrate sometimes caused a partial relaxation, but in other arteries had no clear effect. The mean effect of butyrate on the contracture to 90 mmol l^{-1} K^+, did not achieve significance when the results from 6 similar experiments were combined (see Figure 3).

One explanation for the above results was that butyrate might be acting primarily by causing membrane hyperpolarization, possibly *via* the opening of K^+ channels. In this case, it

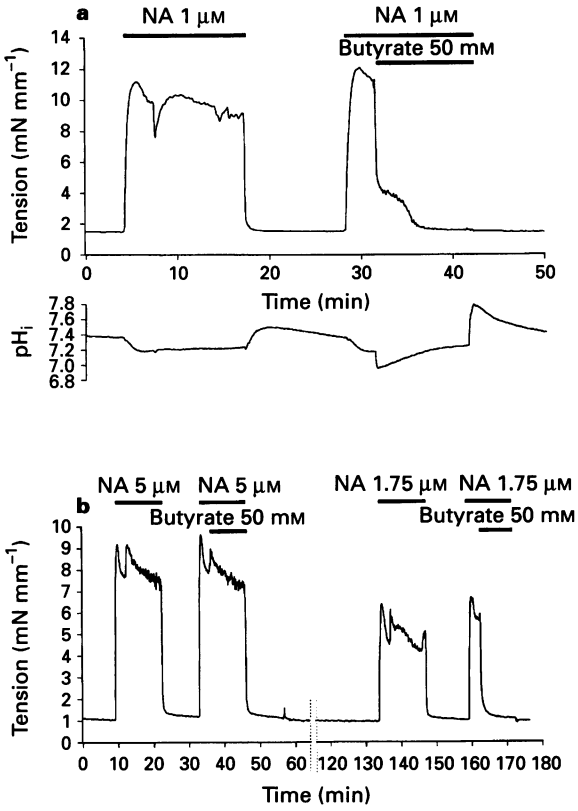

Figure 1 (a) Upper panel, contractures evoked by 1 μmol l^{-1} NA, in the absence of butyrate (left, the NA containing solution was refreshed after 3 min, producing a transient relaxation), and when 50 mmol l^{-1} butyrate was added (in the continuing presence of NA) 3 min after the initial application of NA. Lower panel, simultaneous changes in pH_i, measured by use of BCECF. (b) Effect of 50 mmol l^{-1} butyrate upon the maximal (5 μmol l^{-1} agonist) and approximately half-maximal (1.75 μmol l^{-1} agonist) NA contractures. In both cases, the solution containing butyrate and NA was added 3 min after initial stimulation. Butyrate relaxed the contracture evoked by 1.75, but not 5 μmol l^{-1}, NA.

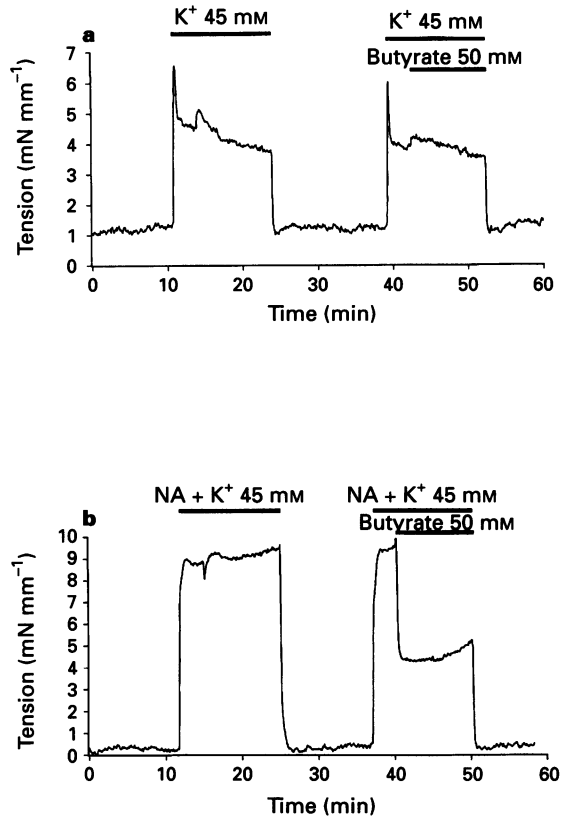

Figure 2 (a) Contractures evoked by 45 mmol l^{-1} K^+ solution (KCl substituted isosmotically for NaCl) in the absence of butyrate (left) and when 50 mmol l^{-1} butyrate was added (in the continuing presence of 45 mmol l^{-1} K^+) 3 min after the start of stimulation (right). Butyrate had no obvious effect upon the high K^+ contracture. (b) The contracture elicited by the combination of 45 mmol l^{-1} K^+ and the approximately half-maximally effective concentration of NA in the absence (left) and presence of 50 mmol l^{-1} butyrate, added 3 min after stimulation was initiated (right).

might be predicted that butyrate would become less effective when the stimulus caused a marked depolarization (e.g. the maximal NA concentration) or both depolarized and 'clamped' the membrane potential (both concentrations of high K^+). This possibility was, however, not consistent with the subsequent observation that butyrate markedly relaxed contractures evoked with a combination of 45 mmol l^{-1} K^+ and NA, as illustrated in Figure 2b.

Figure 3 summarises the effects of 50 mmol l^{-1} butyrate on the contractures elicited by NA, high K^+, and the combination of these. For each type of stimulus, the solid columns represent the time control response, which was measured as the change in the amplitude of the contracture between the 3rd and 13th min after the stimulating agent was applied. Thus, in the absence of butyrate, the contracture tended to decay slightly in NA, but increased somewhat in 45 mmol l^{-1} K^+ and when 45 mmol l^{-1} K^+ and NA were added together. The open columns show the change in the amplitude of the contractures between the 3rd and 13th min when butyrate was added at the beginning of this 10 min period. As described above, butyrate caused a significant relaxation when the preconstriction was caused by a submaximal concentration of NA, or by a submaximal NA concentration added together with 45 mmol l^{-1} K^+; the contractures evoked by 5 μmol l^{-1} NA, or by 45 and 90 mmol l^{-1} K^+, were not significantly relaxed by 50 mmol l^{-1} butyrate. In order, therefore, to characterize further the vasorelaxant effect of butyrate, we subsequently

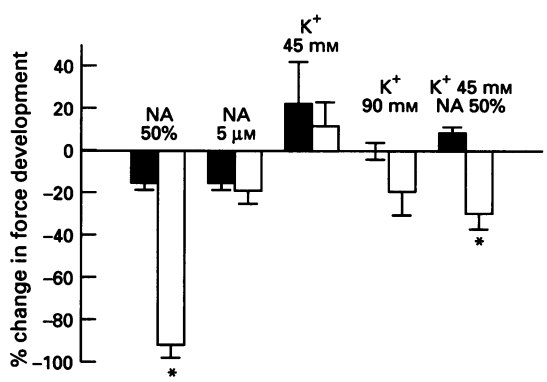

Figure 3 Summary of the effect of 50 mmol l^{-1} butyrate on contractures evoked by NA, high K^+ solution, and their combination. NA 50% indicates that the concentration of NA used was that producing an approximately half-maximal contracture. The ordinate scale represents the % change in the amplitude of the contracture between the 3rd and 13th min of contracture, with the lower section of the axis representing relaxation, and the upper section representing contraction. The solid columns show the intrinsic decay or further development of the contracture over this time period measured in the absence of butyrate. The open columns show how the response changed over this time period if 50 mmol l^{-1} butyrate was applied in the presence of the stimulating agent(s). The asterisks show where butyrate caused a significant ($P<0.05$) reduction in the response, representing a relaxation of the contracture.

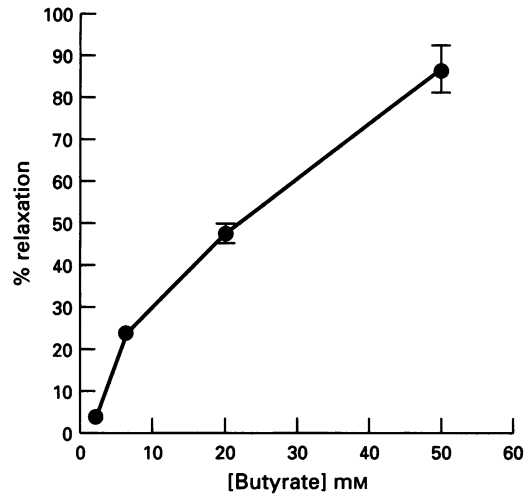

Figure 4 Concentration-response relationship for the butyrate induced relaxation of the half maximal NA contracture. Data were corrected for the intrinsic decay of the NA contracture in each artery, which was measured during a time control contracture. 100% represents the complete relaxation of the contracture during the 10 min period of butyrate application.

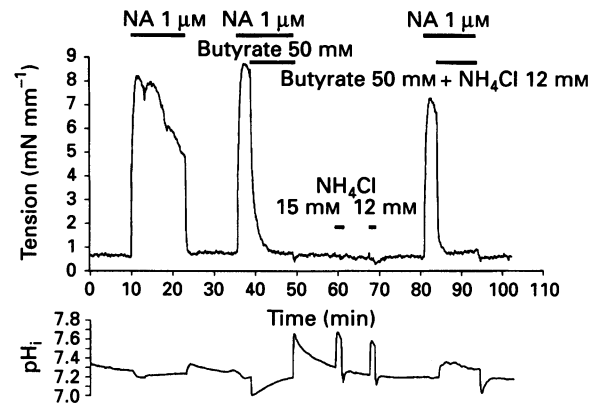

Figure 5 Effect of 'clamping' pH_i on butyrate-mediated relaxation. Application of 50 mmol l^{-1} butyrate caused a typical relaxation of the contracture to 1 μmol l^{-1} NA (compare middle contracture with the time control contracture shown on the left). Addition of 15 and 12 mmol l^{-1} NH_4Cl to the bathing solution was then shown to cause a rapid and reversible increase in pH_i. During the final NA contracture, 50 mmol l^{-1} butyrate and 12 mmol l^{-1} NH_4Cl were added simultaneously, producing a typical relaxation, associated in this case with an alkalinization produced by the NH_4Cl.

focussed upon its effect in arteries preconstricted with a half-maximally effective concentration of NA, since the relaxation obtained was both consistent and marked.

The butyrate concentration-response relationship for the relaxation of the half-maximal NA contracture is illustrated in Figure 4. All relaxations were corrected for the intrinsic decay of the NA response, measured during time controls. NA-induced tension was significantly reduced by 2 mmol l^{-1} butyrate, and inhibited by 24% in 6 mmol l^{-1} butyrate. Half relaxation of the NA contracture was calculated to occur at approximately 22 mmol l^{-1} butyrate.

Use of NH_4Cl to clamp pH_i during butyrate application

In order to determine whether a decrease in pH_i played a role in the butyrate mediated relaxation, NH_4Cl was used to offset the butyrate-mediated acidification, using the protocol shown in Figure 5. An initial estimate of the pH_i change (estimated as the change in the BCECF 490:430 ratio) effected by the addition of 50 mmol l^{-1} butyrate during the NA contracture was made. After butyrate and NA were washed out, various concentrations of NH_4Cl were added to the bath until a concentration which produced an alkalinization (i.e. shift in the BCECF 490:430 ratio) equal to or greater than the weak acid induced acidification was found. Tissues were then preconstricted with NA for 3 min, and butyrate was added together with the appropriate concentration of NH_4Cl. Using NH_4Cl the maximum relaxation to the weak acid was not significantly affected, (97.8±0.7% without NH_4Cl *vs* 88.8±4.9% with NH_4Cl, NS; time controls with neither NH_4 nor butyrate 29.9±7.8%, $n=5$), even though the acidification was prevented or reversed (Figure 5). The steep transient drop in pH_i occurring immediately after application of butyrate was abolished by the inclusion of NH_4Cl in the bath solution. In the absence of NH_4Cl, pH_i in these arteries was 7.25±0.05 immediately before the application of butyrate, and was 7.21±0.04 when relaxation was measured 10 min after application. In the presence of NH_4Cl, however, pH_i was 7.21±0.04 just prior to butyrate addition, and 7.26±0.06 10 min after its application ($n=5$).

Role of the endothelium in butyrate-mediated relaxation

In order to assess the possible contribution of the endothelium to butyrate-induced relaxation, the effect of butyrate was measured both in endothelium-denuded arteries, and endothelium-intact arteries treated with L-NAME. In the former experiments, relaxation of the NA response elicited by 50 mmol l^{-1} butyrate was 88.6±3.8% prior to, and 92.0±4.1% following, removal of the endothelium ($n=3$, NS). In the latter experiments, blockade of nitric oxide synthase with 100 μmol l^{-1} L-NAME (application initiated 20 min prior to NA addition) also did not affect the butyrate-mediated vasorelaxation, which amounted to 87.4±4.3% before L-NAME application, and to 86.9±5.1% in the presence of L-NAME ($n=3$, NS).

Effect of R_p-cAMPS on the butyrate mediated relaxation

R_p-cAMPS is a membrane permeable and biologically inactive derivative of cyclic AMP which competes with cyclic AMP for its binding site on protein kinase A (Van Haastert *et al.*, 1984), thus inhibiting the effect of agents which stimulate cyclic AMP production. We have previously shown, for example, that R_p-cAMPS significantly attenuates the forskolin-mediated relaxation of the NA contracture in these arteries (McKinnon *et al.*, 1996). We therefore examined the effect of R_p-cAMPS on the butyrate-mediated relaxation in order to evaluate the possible role of cyclic AMP in the response to butyrate. Preincubation of the arteries with 25 μmol l^{-1} R_p-cAMPS for 25 min before NA addition markedly reduced the butyrate-induced vasorelaxation (Figure 6). Relaxation measured after 10 min amounted to 92.5±4.3% in 50 mmol l^{-1} butyrate alone, but was reduced to 23.2±20.5% ($n=6$, $P<0.02$) in R_p-cAMPS. This was not significantly different from the 12.8±17.0% relaxation observed in the time controls. The increased variability observed in the presence of R_p-cAMPS was due mainly to the fact that this agent did not affect the maximal extent of the butyrate-mediated relaxation in one of the six arteries examined, although a marked slowing of relaxation did occur in this artery. R_p-cAMPS did not itself cause tension development and had no significant effect on the amplitude of the NA contracture.

Discussion

Butyrate has previously been shown to act as a vasodilator in a number of arteries, including the rat caudal artery (Nutting *et al.*, 1991), the rat mesenteric artery (Austin & Wray, 1993; 1994), and human colonic resistance artery (Mortensen *et al.*, 1990). The concentration-dependency of the butyrate-induced

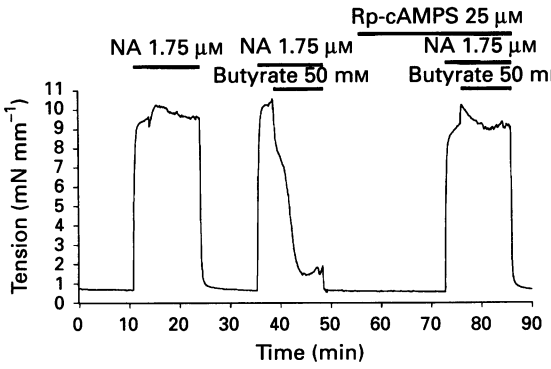

Figure 6 Abolition of the butyrate-mediated relaxation of the NA contracture by 25 μmol l^{-1} $\mathbf{R_p}$-cAMPS. Following a demonstration that 50 mmol l^{-1} butyrate caused a typical relaxation of the NA contracture in this artery, $\mathbf{R_p}$-cAMPS was added to the bathing solution. The artery was restimulated with NA 20 min later in the continuing presence of $\mathbf{R_p}$-cAMPS. Addition of butyrate under these conditions did not lead to a relaxation.

relaxation of the NA contracture measured in the present study is similar to that observed for the relaxation of the phenylephrine-induced contracture in rat caudal artery (Nutting *et al.*, 1991).

A number of different mechanisms have been proposed to explain vasorelaxation by butyrate and other similar SCFA. Application of butyrate causes a decrease in intracellular pH which has been proposed as the main factor in causing relaxation (Austin & Wray, 1993; 1994). However, intracellular acidosis has been shown to have many effects, not all of which would be expected to cause vasorelaxation. Those which should promote vascular relaxation include reduction of the voltage-gated Ca^{2+} current associated with a reduction of Ca^{2+} channel availability (Klöckner & Isenberg, 1994), and stimulation of Ca^{2+} sequestration (Loutzenhiser *et al.*, 1990). On the other hand, rapid acidification causes an increase in tension which is transient or decays with time, (Spurway & Wray, 1986; Aalkjaer & Mulvany, 1988; Matthews *et al.*, 1992), possibly by releasing or displacing intracellular Ca^{2+} (Jensen *et al.*, 1993; Batlle *et al.*, 1993). Proprionate (presumably working *via* intracellular acidification) caused a sustained enhancement of the contraction and Ca^{2+} rise caused by high K^+ depolarization in rabbit portal vein (Iino *et al.*, 1994), even though it inhibited the Ca^{2+} current in cells isolated from the same preparation. Studies in skinned vascular fibres suggest that reduction of pH causes complex, time-dependent effects on the Ca^{2+} *vs* force relationship of the contractile apparatus which vary between different blood vessels (Crichton *et al.*, 1994).

However, in the present study it was possible to dissociate the vasorelaxant effect of butyrate from the cellular acidosis it caused. Firstly, the butyrate-induced acidosis was typically transient (e.g. see Figures 1a and 5), such that pH_i progressively returned to prebutyrate levels within 10 min, even though vasorelaxation was well maintained. Secondly, the butyrate-induced vasorelaxation was not significantly diminished when NH_4Cl was simultaneously applied. Under these conditions, pH_i remained constant or rose slightly, presumably since NH_4Cl entered cells to cause an alkalosis (Spurway & Wray, 1986), which countered the butyrate-mediated acidosis. Although NH_4 in the concentration range used in the present experiments itself causes a brief relaxation in these arteries when applied during a submaximal NA contracture, this transient effect is followed within 2 min by a more persistent potentiation of the NA contracture (Matthews *et al.*, 1992). Thus NH_4Cl could not have contributed to the butyrate-mediated relaxation measured 10 min after these agents were applied.

This result is in accord with our recent observation that the SCFA lactate also caused a marked pH-independent vasorelaxation in these arteries (McKinnon *et al.*, 1996), and also with the earlier finding that raising CO_2 also led to a sustained relaxation of these arteries, while causing only a transient acidosis (Carr *et al.*, 1993).

We have recently shown that relaxation of rat mesenteric small arteries to elevation of the P_{CO_2}, which was associated with a fall in pH_i, did not occur when the endothelium was removed or in the presence of a nitric oxide synthase inhibitor, despite an identical fall in pH_i (Carr *et al.*, 1993). The conclusion from this study was that hypercapnia caused relaxation through nitric oxide release. In the present study, removal of the endothelium was without effect on relaxation to butyrate and a role for nitric oxide release can therefore be excluded; similar observations have been made by Mortensen *et al.* (1990) and Nutting *et al.* (1991).

A number of previous studies have focussed upon the role of cellular second messenger systems in SCFA-induced vasorelaxation. For example, there is evidence that cyclic GMP may be involved in lactate-induced vasorelaxation in the placental circulation as the soluble guanylyl cyclase inhibitor, methylene blue inhibits relaxation to lactate in human placental arteries, (Omar *et al.*, 1993b). These authors suggested that cyclic GMP synthesis might result indirectly from the conversion of lactate to pyruvate, which results in proton generation. The resultant reduction of NAD was proposed to lead to production of hydrogen peroxide and stimulation of cyclic GMP production. The metabolism of butyrate should also result in the reduction of NAD *via* β-oxidation (Coe *et al.*, 1968; Gurr & Harwood, 1991); stimulation of cyclic GMP production might therefore occur. An important role for cyclic GMP in the butyrate-induced relaxation seems unlikely, however, in that $\mathbf{R_p}$-cAMPS completely abolished this effect of butyrate. It is relevant in this regard that H_2O_2 (Matsuda *et al.*, 1993) has been shown to inhibit, rather than stimulate, adenylyl cyclase, and that butyrate and other SCFA did not increase cyclic GMP in rat caudal artery (Nutting *et al.*, 1991). Instead, the inhibition of the response to butyrate by $\mathbf{R_p}$-cAMPS suggests involvement of cyclic AMP in relaxation. $\mathbf{R_p}$-cAMPS is a non functional competitor of cyclic AMP at its binding site on protein kinase A. $\mathbf{R_p}$-cAMPS is relatively resistant to hydrolysis and so if present in sufficiently high concentrations will block activation of protein kinase A (Van Haastert *et al.*, 1984; de Wit *et al.*, 1984). The effect of $\mathbf{R_p}$-cAMPS is consistent with experiments carried out in cultured vascular smooth muscle from the rat caudal artery (Nutting *et al.*, 1991; 1992) in which intracellular cyclic AMP increased rapidly after exposure to the weak acids, acetate, butyrate and propionate. All three weak acids increased cyclic AMP levels independently of a functional endothelium (Daugirdas *et al.*, 1988; Nutting *et al.*, 1991). The present experiments provide direct evidence that this rise in cyclic AMP does contribute to butyrate-induced relaxation in rat mesenteric arteries. As in the rat caudal artery (Nutting *et al.*, 1992), this effect is unlikely to involve adenosine generated *via* AMP produced during the synthesis of butyryl CoA (Smith *et al.*, 1983), since adenosine has been shown not to raise cyclic AMP concentrations in the rat mesenteric artery (Vuorinen *et al.*, 1992). Any effect of butyrate on adenylyl cyclase is also unlikely to be caused by a decrease in pH_i, since acidification inhibits the activity of this adenylyl cyclase from a variety of tissues (e.g. Johnson, 1982).

The mechanism by which butyrate increases cyclic AMP remains unknown. However, it has been reported in rat hepatocytes that a rise in the [NADH]/[NAD^+] ratio increased glucagon-stimulated cyclic AMP production by inhibiting phosphodiesterase (Clark & Jarrett, 1976). It is possible, therefore, that extramitochondrial (peroxisomal) β-oxidation of butyrate in these cells might increase NADH sufficiently to promote cyclic AMP accumulation *via* a similar effect.

We cannot at present explain the observation that butyrate causes little relaxation of the high K^+ contracture. However, its lack of effect does not appear to be due to the membrane

depolarization which occurs in high K^+, since the contracture evoked by a combination of NA and high K^+ was markedly relaxed by butyrate. Nonetheless, our data indicate that the inhibition of Ca^{2+} influx *via* voltage-gated Ca^{2+} channels is not the basis of the effect of butyrate.

It is noteworthy that our results differ from those reported recently by Austin & Wray (1994), who found that butyrate relaxed high K^+ depolarized rat mesenteric resistance arteries of similar diameter to those we studied. However, the magnitude of relaxation of the high K^+ contracture which they described was much smaller than the relaxation of the NA contracture which we have reported; 40 mmol l^{-1} butyrate relaxed the high K^+ contracture by less than 30%. Conversely, our butyrate concentration-response relationship suggested that 40 mmol l^{-1} butyrate would relax the response to NA by more than 70%. We did find that 50 mmol l^{-1} butyrate sometimes caused an obvious but limited relaxation of the high K^+ contracture, especially when 90 mmol l^{-1} K^+ was used, although this effect was much smaller than the relaxation of the NA contracture which was obtained in the same arteries. Previous studies in these arteries demonstrated that the α-adrenoceptor antagonist, phentolamine, reduced the amplitude of the contracture to 90 mmol l^{-1} K^+ by more than half (Graves & Poston, 1993); a slightly smaller effect of phentolamine was observed at 45 mmol l^{-1} K^+. A substantial fraction of the high K^+ contracture under these conditions is therefore due to the release of NA from sympathetic nerve varicosities within the arteries. It is possible, therefore, that the effect of butyrate on the high K^+ contracture might have been due to suppression of this NA-mediated component of the contracture. This possibility, however, requires experimental confirmation.

In summary, the present observations suggest that butyrate causes relaxation of the NA contracture through a pH_i- and endothelium-independent pathway which also does not involve the inhibition of voltage-gated Ca^{2+} channels. Rather, the blockade of this response by R_p-cAMPS confirms and extends previous observations (Nutting *et al.*, 1991; 1992) that weak acid induced relaxation depends to an important degree on a stimulation of protein kinase A *via* a rise in cyclic AMP.

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