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Influence of increase in osmotic pressure with sucrose on relaxation and cyclonucleotides levels in isolated rat aorta

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Abstract

The influence of increases in osmolarity by addition of sucrose were investigated on relaxation and changes in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels in isolated rat aortic rings. Isoprenaline-mediated relaxations were attenuated in hypertonic (341 ± 0.4 mOsmol) (mean ± S.E.M.) solution. The concentration–response curve to isoprenaline was displaced to the right. The EC_{50} (0.16 ± 0.05 vs. 1.14 ± 0.5 μM) significantly ($n = 6$; $P < 0.05$) increased without any changes to the maximum response. Hypertonic solution also attenuated methacholine-mediated relaxations resulting in a significant increase in the EC_{50} (0.28 ± 0.04 vs. 0.52 ± 0.04 μM) and reduced the maximal response (73 ± 5% vs. 51 ± 8%). In contrast, an increase in tonicity did not have any influence on sodium nitroprusside, forskolin or pinacidil concentration–response curves. Hypertonic solution also did not affect either basal cyclic AMP or cyclic GMP production. In addition, an increase in osmolarity did not affect isoprenaline-stimulated increases in the levels of cyclic AMP. However, an increase in the tonicity of Krebs solution significantly inhibited methacholine-stimulated (58%–34%) accumulation of cyclic GMP. The present data indicated that an increase in the tonicity of Krebs solution impaired endothelium-dependent relaxation and the associated increase in cyclic GMP production without affecting basal levels of this nucleotide. The inhibitory effects of high osmolarity on β-adrenoceptor-mediated relaxation did not appear to be due to a reduction in cyclic AMP generation, or the result of inhibition of pinacidil-sensitive K^+_{ATP} channels. Moreover, an increase in the tonicity of Krebs solution did not influence relaxation induced by direct activation of adenylate cyclase or guanylate cyclase by forskolin and sodium nitroprusside, respectively.

Keywords: Aortic ring, rat; Relaxation; Cyclonucleotide; Hypertonicity; Isoprenaline; Methacholine

1. Introduction

An increase in the tonicity of physiological salt solution appears to produce a varied effect on the functional behavior of isolated blood vessels. For instance, an increase in the osmolarity of physiological salt solution with sucrose reportedly results in the contraction of isolated blood vessels (Andersson et al., 1974; Sato et al., 1994). In contrast, hypertonic solution containing sucrose has also been reported to inhibit agonist-mediated contraction in isolated blood vessels (Krishnamurty et al., 1977). Moreover, in studies in which the effects of hypertonic sucrose solution were examined on the spontaneous contractions of rat isolated portal vein, it was reported that an increase in the tonicity of physiological salt solution reduced the frequency of contraction without affecting the amplitude of the spontaneous response (Johansson and Jonsson, 1968). Certainly, large increases in the tonicity of physiological salt solution by the addition of sucrose (~ 500 mOsmol) results in (a) contraction of vascular smooth muscle, (b) augmentation of K^+- or Ba^{2+}-induced contractions, (c) partial inhibition of noradrenaline-induced contractions, and (d) inhibition of neuronal release of noradrenaline in isolated strips of dogs' saphenous veins (McGrath and Shepherd, 1976). It seems that exposure of isolated blood vessels to hypertonic solution initially (within 1 min) attenuates contraction to agonists such as noradrenaline and angiotensin II but that this effect is followed (within 25–30 min) by potentiation of the contractile responses to these agonists (Blair-West et al., 1971; Mckinley et al., 1974).

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Shrinkage of smooth muscle cells has been observed in sucrose-hypertonic solution (150 mM) after 20 min, and sucrose has been noted to diffuse into the extracellular space with very limited entrance into the intracellular space (Arvill et al., 1969). In addition, large increases in the tonicity of physiological salt solution with sucrose (~ 580 mOsmol) for a period of 1 h has been shown to result in the extensive dehydration of smooth muscle cells, associated with extensive cell shrinkage (Kirkpatrick et al., 1980). However, it is believed that active contraction rather than passive shrinkage appears to be responsible for the increase in tension observed in isolated blood vessel in response to changes in extracellular tonicity (Andersson et al., 1974). Hypertonic solution also has an impact on endothelial cells, and cell shrinkage has been found to occur following exposure to hypertonic solutions (Mazzoni et al., 1989). It is well recognized that changes in the endothelial cell volume has an affect on the biophysical and functional properties of these cells (Fransen et al., 1995; Klein and O'Neill, 1995; Nilius et al., 1997, 1998). Therefore, the direct effect that an increased osmolarity of extracellular fluid may have on smooth muscle function will undoubtedly also be influenced by changes in the function of endothelial cells.

At present, there does not appear to be any evidence in the published literature of investigations that have examined the influence of an increase in osmolarity on relaxation in isolated blood vessels. Thus, the influence of changes in osmolarity on vascular relaxation remains to be investigated. In the present investigation, we have attempted to examine the influence of moderate increases in osmolarity by addition of sucrose on (a) adrenoceptor- and cholinoceptor-mediated relaxations, (b) basal and agonist-mediated changes in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels, and (c) actions of other vasodilators, namely, sodium nitroprusside, forskolin and pinacidil in isolated rat aortic rings.

2. Methods

2.1. Tissue isolation

Male Sprague–Dawley rats (230–260 g) were anaesthetized with sodium pentobarbital (65 mg/kg i.p.). The thoracic aorta was removed and dissected free of connective tissue at room temperature in Krebs buffer of the following composition (in mM): NaCl, 120; KCl, 4.6; glucose, 11; $MgCl_2$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.3. The pH of the buffer following saturation with a 95% O_2:5% CO_2 gas mixture was 7.4. Experiments were performed both in regular Krebs solution of osmolarity 301 ± 0.3 mOsmol ($n = 30$, mean ± S.E.M.) and in hypertonic solutions prepared by the addition of sucrose, at the following concentrations (in mM): 39, 53 and 71, to regular Krebs. The osmolarity of each Krebs solution containing sucrose (39, 53 and 71 mM) was 320 ± 0.5 mOsmol ($n = 6$; mean ± S.E.M.), 331 ± 0.6 mOsmol ($n = 6$; mean ± S.E.M.), and 341 ± 0.4 mOsmol ($n = 24$; mean ± S.E.M.), respectively. The osmolarity of all solutions was measured by an Osmometer (Precision Systems Model No. 5004, Fisher Scientific, ON, Canada) using the freezing point depression method.

2.2. Relaxation studies

Aortic rings (~ 2 mm in length) were mounted in 20 ml organ baths at 37°C under a force of 19.6 mN and gassed continuously with a mixture of 95% O_2:5% CO_2. The tissues were equilibrated for 60 min and isometric tension was measured using force displacement transducers (Model FT03, Grass Instruments, MA, USA) connected to a polygraph (Model 7PCPB, Grass Instruments). Tissues were initially contracted with phenylephrine (1.0 μM) and then relaxed with a single concentration of isoprenaline (0.3 μM), methacholine (0.3 μM), sodium nitroprusside (0.1 μM), forskolin (0.3 μM) or pinacidil (1.0 μM) in regular Krebs solution. Tissues were washed with regular Krebs solution and left for an additional 60 min (washed once at 30 min with regular Krebs) before they were contracted by phenylephrine (1.0 μM) and a control cumulative concentration–response curve to each agonist, isoprenaline (1.0 nM–10 μM), methacholine (10 nM–10 μM), sodium nitroprusside (1.0 nM–1.0 μM), forskolin (10 nM–1.0 μM) or pinacidil (100 nM–30 μM) was constructed. The tissues were then washed with regular Krebs and allowed to equilibrate for 30 min. After this period, each tissue was incubated with a sucrose-hypertonic solution or regular Krebs solution (time-control) for 30 min. Subsequently, tissues were contracted with phenylephrine (1.0 μM) and a second cumulative concentration–response curve to each agonist (isoprenaline, methacholine, sodium nitroprusside, forskolin or pinacidil) was constructed. For isoprenaline and methacholine, a third concentration–response was constructed after tissues were returned to regular Krebs solution (60 min after the construction of the second concentration–response curves). In addition, two groups of experiments were conducted in the presence of the phosphodiesterase inhibitor, pentoxifylline (30 μM), in hypertonic solution. After the completion of the control concentration–response curves to isoprenaline and methacholine, tissues were incubated in sucrose-hypertonic solution (341 ± 0.4 mOsmol) as previously described. Twenty minutes before the tissues were to be contracted, pentoxifylline was added. A cumulative concentration–response curve was then carried out for isoprenaline or methacholine in the presence of pentoxifylline in hypertonic solution.

2.3. Cyclonucleotide studies

Aortic rings (~ 4 mm in length) were incubated in 125 ml of normal Krebs solution for 90 min and gassed

Table 1
EC_{50}, Hill coefficient (n_H) and percent maximum response values from individual concentration–response curves under different osmotic conditions in aortic ring preparations

Group (mOsmol)	EC_{50} (μM)	n_H	% maximum
Isoprenaline			
301 ± 0.3	0.18 ± 0.03	1.2 ± 0.1	85 ± 5
321 ± 0.5	0.21 ± 0.06	1.2 ± 0.1	89 ± 4
301 ± 0.3	0.29 ± 0.10	1.2 ± 0.1	90 ± 3
331 ± 0.6	0.61 ± 0.20[a]	1.2 ± 0.1	90 ± 4
301 ± 0.3	0.31 ± 0.10	1.4 ± 0.2	82 ± 3
301 ± 0.3	0.16 ± 0.05	1.4 ± 0.3	90 ± 3
341 ± 0.4	1.14 ± 0.5[a]	1.3 ± 0.1	94 ± 2
301 ± 0.3	0.12 ± 0.05	1.2 ± 0.1	95 ± 2
301 ± 0.3	0.26 ± 0.08	1.3 ± 0.1	84 ± 5
341 ± 0.4 + pentoxi (30 μM)	0.28 ± 0.1	1.5 ± 0.3	86 ± 3
301 ± 0.3	0.23 ± 0.09	1.2 ± 0.1	89 ± 7
301 ± 0.3	0.16 ± 0.04	1.3 ± 0.1	88 ± 3
301 ± 0.3	0.15 ± 0.02	1.4 ± 0.2	81 ± 3
Methacholine			
301 ± 0.3	0.28 ± 0.02	1.6 ± 0.1	73 ± 6
321 ± 0.5	0.35 ± 0.03	1.6 ± 0.1	70 ± 7
301 ± 0.3	0.29 ± 0.08	1.9 ± 0.3	82 ± 6
331 ± 0.6	0.47 ± 0.09[a]	2.0 ± 0.3	69 ± 4
301 ± 0.3	0.43 ± 0.08[a]	1.7 ± 0.1	80 ± 6
301 ± 0.3	0.28 ± 0.04	1.6 ± 0.2	73 ± 5
341 ± 0.4	0.52 ± 0.04[a]	2.0 ± 0.2	51 ± 8[a]
301 ± 0.3	0.40 ± 0.03[a]	1.6 ± 0.1	72 ± 6
301 ± 0.3	0.22 ± 0.05	1.9 ± 0.2	70 ± 7
341 ± 0.4 + pentoxi (30 μM)	0.24 ± 0.02	2.0 ± 0.1	70 ± 9
301 ± 0.3	0.29 ± 0.06	1.9 ± 0.1	82 ± 6
301 ± 0.3	0.29 ± 0.06	1.8 ± 0.1	84 ± 6
301 ± 0.3	0.30 ± 0.05	1.8 ± 0.1	78 ± 7

[a]Significantly different from regular Krebs solution (301 ± 0.3 mOsmol) ($P < 0.05$). Data represents means ± S.E.M.

continuously with a 95% O_2:5% CO_2 gas mixture kept at 37°C. The buffer was replaced with fresh buffer every 30 min. Each tissue was then transferred into scintillation vials containing 10 ml of regular Krebs solution and allowed to equilibrate for 20 min at 37°C while gassed continuously with 95% O_2:5% CO_2. The physiological salt solution was then replaced with 10 ml of either normal buffer or sucrose hypertonic buffer (341 ± 0.4 mOsmol) and allowed to equilibrate for 20–25 min. Following equilibration, either double distilled water (10 μl), isoprenaline (0.1–3.0 μM) or methacholine (0.1–3.0 μM) was added to the tissues. Drugs or distilled water were allowed to be in contact with each tissue for 5 min. Subsequently, each tissue was rapidly frozen in liquid nitrogen and stored at −80°C until it was assayed for cyclic nucleotides.

Cyclic nucleotides in aortic rings were measured by the technique described by Delpy et al. (1996). Briefly, frozen aortic rings were homogenized in 0.4 ml of cold trichloroacetic acid (6%) using an electric tissue homogenizer (Dyna-mix Model 43, Fisher Scientific). The homogenate was centrifuged using an eppendorf centrifuge (Model 5415C, Brinkman Instruments, NY, USA) at 5000 × *g* for approximately 1 min and the supernatant was decanted without disturbing the protein pellet. Subsequently, the supernatant was washed four times with 6 volumes of water saturated diethyl ether to remove the trichloroacetic acid. The upper ether layer was discarded after each wash. Following the final extraction, any remaining ether was evaporated under air stream. Throughout these procedures, the samples were consistently stored at approximately 4°C in an ice–water bath. The aqueous phase was then evaporated to dryness with a Speed-vac system (Model SVC100H, Savant Instruments, London, UK) for approximately 1.5 h and the dried extract dissolved in a suitable volume of Tris–EDTA assay buffer prior to analysis. Cyclic AMP and cyclic GMP content was determined by scintillation proximity assay kits (Amersham, ON, Canada). Protein concentration in the pellet was measured using the method of Bradford (1976) (Bio-Rad Life Research Product, ON, Canada).

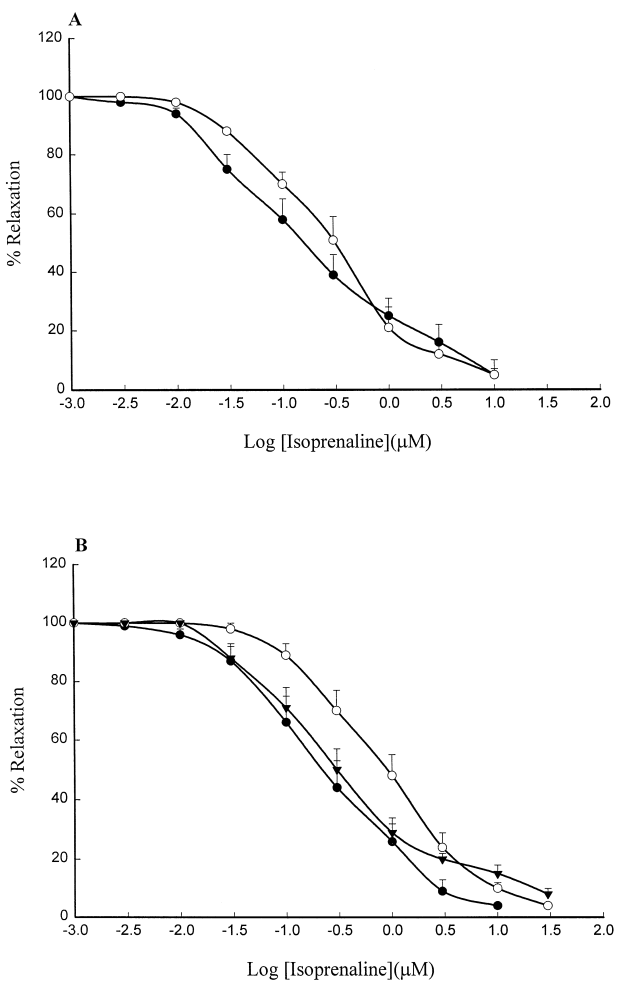

Fig. 1. Concentration–response curves to isoprenaline in (A) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles) and sucrose-hypertonic solution (321 ± 0.5 mOsmol) (open circles); (B) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), sucrose-hypertonic solution (331 ± 0.6 mOsmol) (open circles), and subsequently again in regular Krebs solution (301 ± 0.3 mOsmol) (closed triangles). $n = 6$; mean ± S.E.M.

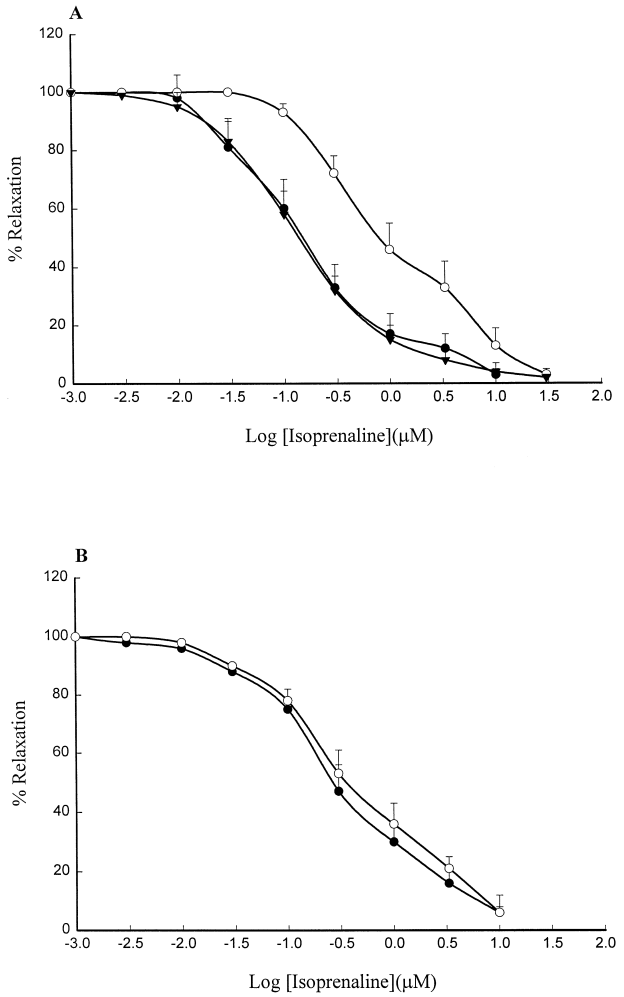

Fig. 2. Concentration–response curves to isoprenaline in (A) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles), and subsequently again in regular Krebs solution (301 ± 0.3 mOsmol) (closed triangles); (B) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), and sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles) in the presence of pentoxifylline (30 μM). $n = 6$; mean ± S.E.M.

2.4. Data and statistical analysis

Results from the relaxation studies were calculated as a percentage of the maximum relaxation induced by agonists following contraction with phenylephrine. Percent maximum, Hill coefficient (n_H) and EC_{50} values were calculated for individual curves using a program executed on an IBM compatible microcomputer (Wang and Pang, 1993). These parameters were determined by fitting the percent contractile response at increasing concentrations of agonist ([A]) by non-linear least squares to the relation $Y = a + bX$, where Y = response and $X = [A]^n/([A]^n + [EC_{50}]^n)$ with n (n_H) fixed at 'floating' integral values to obtain the best fit. An analysis of variance was used for comparisons of percent maximum, n_H and EC_{50}. For multiple comparisons, Duncan's multiple range test was used to compare between means. Cyclic nucleotide content was calculate as pmoles of cyclic nucleotide per mg of protein and is expressed as change from basal. Unpaired *t*-test was employed for parallel comparisons between cyclic nucleotide levels to compare between means. For all cases, a probability of error of less than 0.05 was selected as the criterion for statistical significance.

2.5. Chemicals

Stock solutions of all drugs except for pinacidil and forskolin were made in double distilled water. Stock solutions of pinacidil (20 mM) and forskolin (20 mM) were prepared in 0.1 M NaOH and dimethyl sulfoxide, respectively, and diluted in double distilled water. Methacholine hydrochloride, pentoxifylline and forskolin were purchased from Sigma (ON, Canada). Isoprenaline hydrochloride,

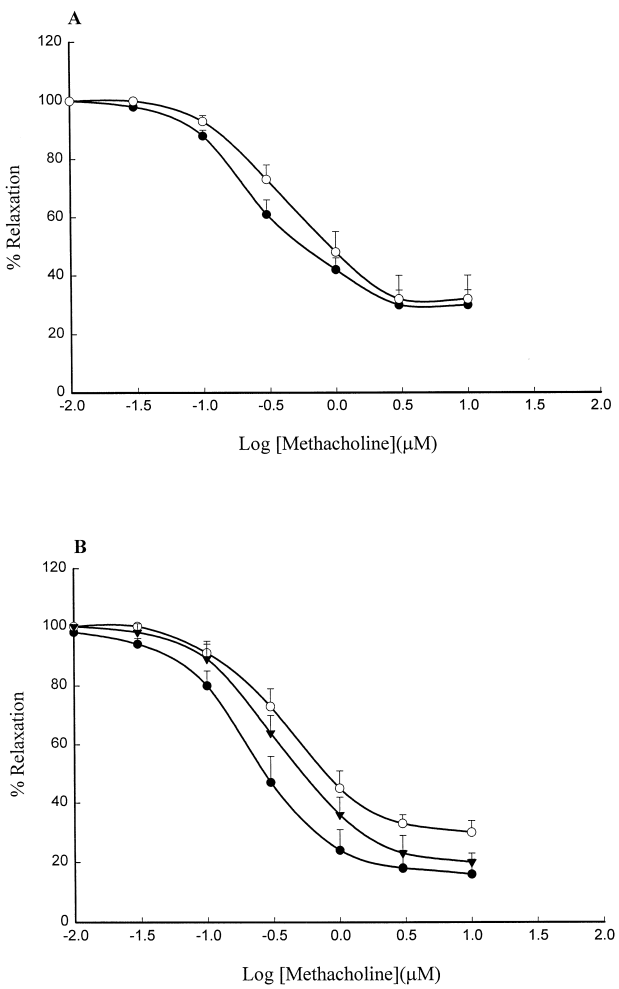

Fig. 3. Concentration–response curves to methacholine in (A) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles) and sucrose-hypertonic solution (321 ± 0.5 mOsmol) (open circles); (B) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), sucrose-hypertonic solution (331 ± 0.6 mOsmol) (open circles), and subsequently again in regular Krebs solution (301 ± 0.3 mOsmol) (closed triangles). $n = 6$; mean ± S.E.M.

sodium nitroprusside and pinacidil were purchased from Research Biochemical International (MA, USA).

3. Results

3.1. Effects of hypertonic solution on isoprenaline- and methacholine-mediated relaxation of isolated aortic rings

An increase in the osmolarity of the physiological salt solution (320 ± 0.5, 331 ± 0.6, 341 ± 0.4 mOsmol) by the addition of sucrose did not affect baseline tension in the tissue preparations. Moreover, the hypertonic solution also did not affect the amplitude of phenylephrine-induced contraction when compared to normal physiological salt solution (301 ± 0.3 mOsmol). An increase in the osmolarity of the solution did attenuate both isoprenaline- and methacholine-mediated relaxation in the aortic ring preparation. The extent of impairment corresponded well with the increase in tonicity of the solution (Table 1).

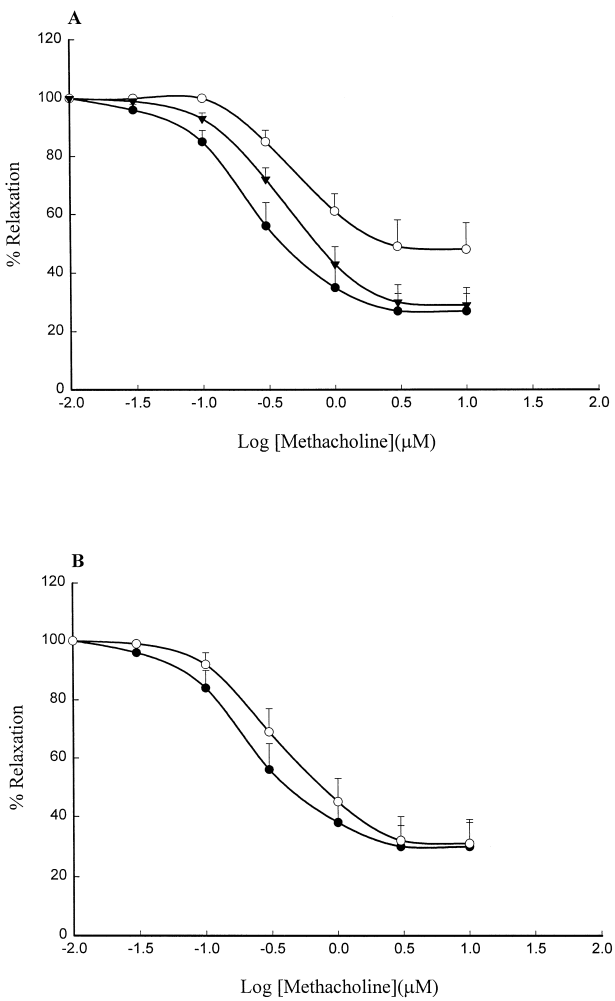

Fig. 4. Concentration–response curves to methacholine in (A) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles), and subsequently again in regular Krebs solution (301 ± 0.3 mOsmol) (closed triangles); (B) regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), and sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles) in the presence of pentoxifylline (30 μM). $n = 6$; mean ± S.E.M.

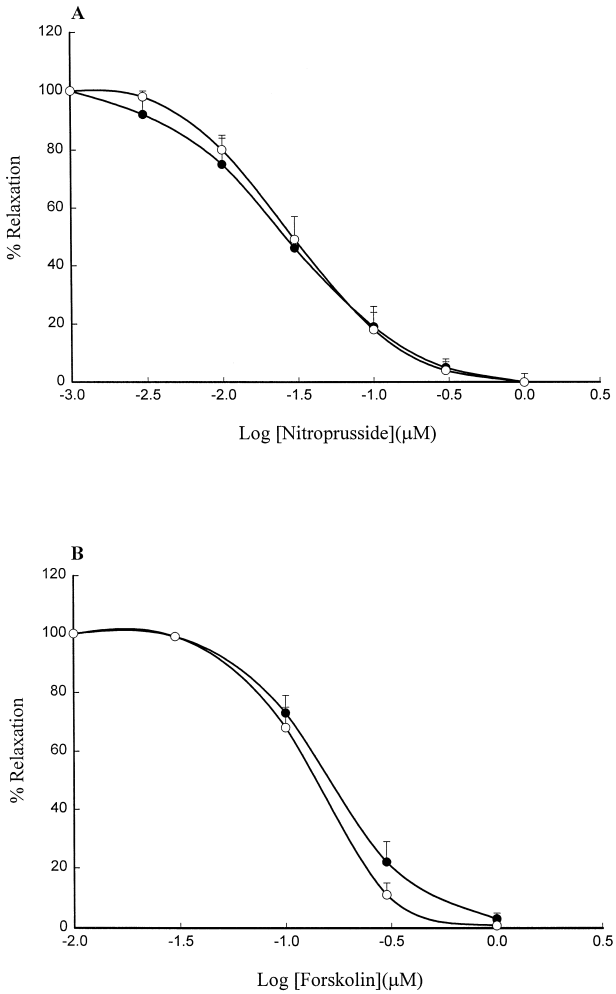

Fig. 5. Concentration–response curves (A) to sodium nitroprusside in regular Krebs solution (301 ± 0.3 mOsmol) (closed circles) and sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles), and (B) to forskolin in regular Krebs solution (301 ± 0.3 mOsmol) (closed circles), sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles). $n = 6$; mean ± S.E.M.

Isoprenaline-induced relaxations were not significantly perturbed at 320 ± 0.5 mOsmol (Fig. 1A). However, an increase in osmolarity to 331 ± 0.6 and 341 ± 0.4 mOsmol resulted in an inhibition of the relaxant response mediated by isoprenaline (Figs. 1B and 2A). The concentration–response curve to isoprenaline was displaced to the right. The EC_{50} was significantly increased without any changes to either the maximum response or the Hill coefficient (Table 1). Moreover, the effects of the hypertonic solution on isoprenaline were reversed following replacement of the hypertonic solution with normal physiological salt solution (Figs. 1B and 2A). In addition, pre-incubation of tissues with the phosphodiesterase inhibitor, pentoxifylline (30 μM), in hypertonic solution (341 ± 0.4 mOsmol) also

reversed the inhibitory effects of the increased tonicity salt solution (Fig. 2B). In time-control studies, reproducible concentration–response curves to isoprenaline were obtained (Table 1).

Methacholine-induced relaxations were also not significantly impaired at the lowest increase in osmolarity (320 ± 0.5 mOsmol) (Fig. 3A). At the two higher osmotic pressures, methacholine-mediated relaxations were found to be attenuated (Figs. 3B and 4A). The concentration–response curves to methacholine were displaced to the right with significant increases in the EC_{50} values. While the maximal responses to methacholine were not reduced by a solution with an osmolarity of 331 ± 0.6 mOsmol, they were significantly reduced at 341 ± 0.4 mOsmol (Table 1). Hypertonic solutions did not affect the Hill coefficients of the concentration–response curves to methacholine. The impact of an increase in osmolarity on methacholine-induced relaxations was not entirely reversed by switching back to normal physiological salt solution (Table 1). The inhibitory effects of the increase in osmolarity (341 ± 0.4 mOsmol) on methacholine-induced relaxation were reversed in the presence of pentoxifylline (30 μM) (Fig. 4B). Concentration–response curves to methacholine over time were reproducible (Table 1).

3.2. Effects of hypertonic solution on sodium nitroprusside-, forskolin- and pinacidil-mediated relaxation in isolated aortic rings

An increase in the osmotic pressure did not have any influence on either sodium nitroprusside or forskolin concentration–response curves (Fig. 5A and B). The relaxant effects of sodium nitroprusside and forskolin were reproduced in time-control studies (Table 2). In contrast, pinacidil-mediated relaxations were significantly attenuated over time (Fig. 6A). Parallel comparisons between concentration–response curves to pinacidil, in terms of EC_{50}, maximal responses and Hill coefficient values, indicated that the hypertonic solution had no significant effect (Fig. 6A and B; Table 2).

Table 2
EC_{50}, Hill coefficient (n_H) and percent maximum response values from individual concentration–response curves under different osmotic conditions in aortic ring preparations

Group (mOsmol)	EC_{50} (μM)	n_H	% maximum
Sodium nitroprusside			
301 ± 0.3	0.037 ± 0.008	1.6 ± 0.2	99 ± 1
301 ± 0.3	0.031 ± 0.004	1.7 ± 0.2	98 ± 1
301 ± 0.3	0.038 ± 0.009	1.6 ± 0.1	98 ± 2
341 ± 0.4	0.036 ± 0.008	1.6 ± 0.2	99 ± 2
Forskolin			
301 ± 0.3	0.22 ± 0.06	2.4 ± 0.2	99 ± 1
301 ± 0.3	0.15 ± 0.03	2.4 ± 0.2	98 ± 1
301 ± 0.3	0.18 ± 0.03	2.5 ± 0.1	99 ± 1
341 ± 0.4	0.15 ± 0.02[a]	2.7 ± 0.2	99 ± 1
Pinacidil			
301 ± 0.3	1.10 ± 0.20	2.6 ± 0.30	91 ± 2
301 ± 0.3	1.99 ± 0.30[a]	1.5 ± 0.20[a]	89 ± 2
301 ± 0.3	1.00 ± 0.16	2.8 ± 0.16	88 ± 2
304 ± 0.4	2.00 ± 0.30[a]	1.5 ± 0.30[a]	91 ± 3

[a] Significantly different from the first response curve ($P < 0.05$). Data represents means ± S.E.M.

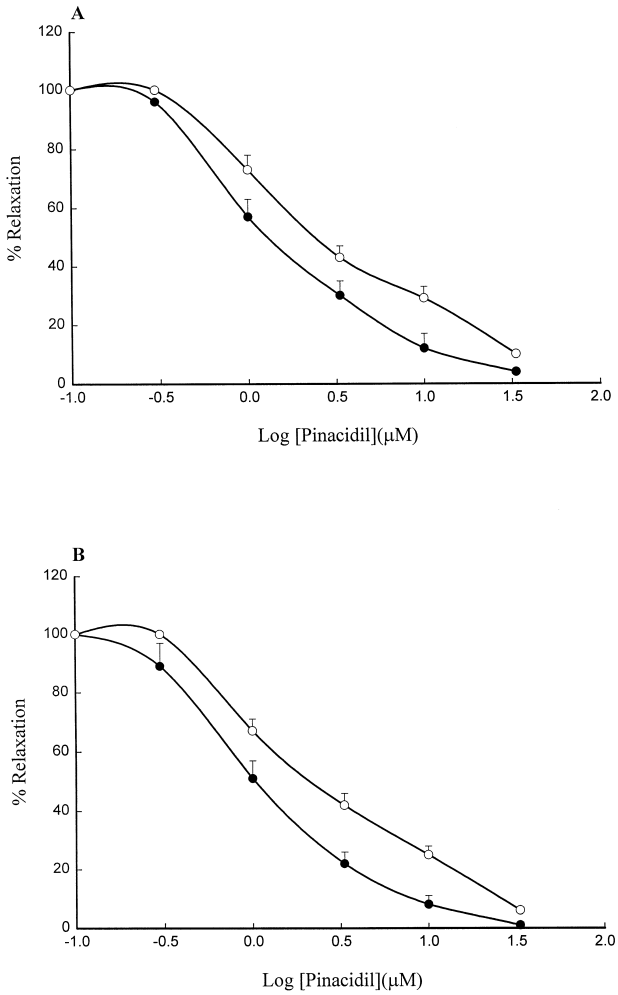

Fig. 6. Concentration–response curves to pinacidil in (A) (time-control) in regular Krebs solution (301 ± 0.3 mOsmol) (closed and open circles), and (B) regular Krebs solution (301 ± 0.3 mOsmol (closed circles), and sucrose-hypertonic solution (341 ± 0.4 mOsmol) (open circles). $n = 6$; mean ± S.E.M.

3.3. Effect of hypertonic solution on basal-, isoprenaline- and methacholine-induced cyclic AMP and cyclic GMP levels in isolated aortic rings

In the presence of isoprenaline, cyclic AMP levels significantly increased (1.5–2.5 fold) above basal levels. A hypertonic solution (341 ± 0.4 mOsmol) did not affect basal cyclic AMP production. In addition, an increase in osmolarity did not affect isoprenaline-induced increases in the levels of cyclic AMP (Fig. 7A).

Methacholine also produced a significant increase (2.2–3.8 fold) above basal cyclic GMP levels. A hypertonic

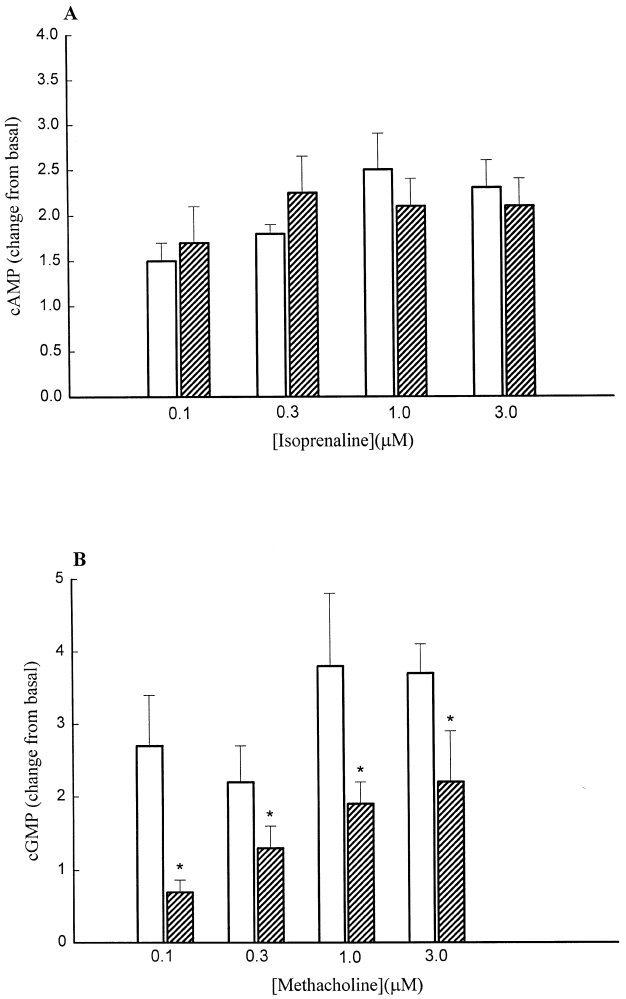

Fig. 7. Change from basal in cyclic nucleotide levels in isolated aortic rings after stimulation with (A) isoprenaline in regular Krebs solution (301 ± 0.3 mOsmol; basal cyclic AMP level 69.5 ± 8.0 pmol mg protein^{-1}) (opened bars), and sucrose-hypertonic solution (341 ± 0.4 mOsmol; basal cyclic AMP level 58.2 ± 14.0 pmol mg protein^{-1}) (hatched bars), or (B) methacholine in regular Krebs solution (301 ± 0.3 mOsmol; basal cyclic GMP level 31.0 ± 7.0 pmol mg protein^{-1}) (opened bars), and sucrose-hypertonic solution (341 ± 0.4 mOsmol; basal cyclic GMP level 43.0 ± 12.0 pmol mg protein^{-1}) (hatched bars). $n = 6$; mean ± S.E.M. * Significantly different from respective values obtained in regular Krebs solution.

solution (341 ± 0.4 mOsmol) did not have any influence on basal cyclic GMP levels. However, an increase in the tonicity of the physiological salt solution significantly attenuated methacholine-induced accumulation of cyclic GMP by $58 \pm 12\%$, $42 \pm 12\%$, $34 \pm 10\%$ and $45 \pm 10\%$ of their respective control at 0.1, 0.3, 1.0 and 3.0 μM, respectively (Fig. 7B).

4. Discussion

An increase in the osmolarity of physiological salt solutions with sucrose has been found to produce shrinkage of vascular smooth muscle cell volume (Johansson and Jonsson, 1968; Arvill et al., 1969). In the present investigation, we find that moderate increases in the tonicity of physiological salt solution with sucrose results in an impairment in the vasorelaxant effects of both isoprenaline and methacholine. However, the evidence also indicates that the vasorelaxant effects of directly acting vasodilators, including sodium nitroprusside, forskolin and pinacidil, are not impaired by an increase in the tonicity of extracellular fluid. In addition, it appears that at the cellular level, the impact of an increase in the tonicity of extracellular fluid on the vasorelaxant effects of isoprenaline vs. methacholine are different.

In simple terms, the influence of increased osmolarity on the relaxant effects of isoprenaline differs from methacholine in two ways. First, it is apparent that the maximal response to the effects of methacholine is reduced by an increase in the tonicity of the physiological salt solution. This is not the case for isoprenaline since the maximal response was retained in hypertonic solution. Secondly, the inhibitory effect of the hypertonic solution on isoprenaline-induced relaxations is completely reversible once the tissues are returned to isotonic buffer, and this is not entirely so for methacholine. It is apparent that after the replacement of hypertonic solution with regular Krebs solution, methacholine-mediated relaxation does not completely revert back to normal. Certainly, the possibility of endothelial cell damage has to be considered. This possibility appears unlikely as the basal levels of cyclic GMP did not appear to be significantly affected in hypertonic solution. However, alteration in ion fluxes as a result of increased tonicity of physiological salt solution may have contributed to changes in second messenger responses to methacholine. It is recognized, that exposure of endothelial cells to hypertonic solution produces shrinkage of these cells (Mazzoni et al., 1989; Perry and O'Neill, 1994). Moreover, it has been suggested that hypertonic solution can result in inactivation of Ca^{2+}-dependent K^+ channels (Perry and O'Neill, 1994). In addition, evidence in the literature indicates that changes in osmolarity with sucrose, within the same range as that in the present study, result in an increased influx of K^+ into the cell (O'Donnell, 1993). In fact, exposure of endothelial cells to sucrose-hypertonic solution seems to result in an increase in the intracellular Na^+ and K^+ content of the cells (O'Donnell, 1993). Simply put, it is most likely that alterations in the intracellular milieu of the endothelial cells in hypertonic solution played a role in impairing the actions of methacholine in generating normal levels of second messenger(s) to produce a response.

It is quite clear that the reduction in relaxant responses to methacholine in the hypertonic solution, at least in part, is related to reduced production of cyclic GMP since we find that the hypertonic solution significantly reduced methacholine-stimulated cyclic GMP production in isolated blood vessels. This may argue for a mechanism that

is associated with a dysfunctional agonist–receptor system under conditions where the tonicity of the extracellular fluid exceeds a certain level above the norm. However, it must be stated that the calculated Hill coefficient was not significantly changed in hypertonic vs. regular Krebs solution for methacholine. Perhaps this indicates that agonist–receptor dynamics were not drastically altered in hypertonic solution. Nonetheless, this does not rule out the possibility of some problem with the efficiency of agonist to generate a complete signal. It is also quite clear that the effects of hypertonic solution were reversed in the presence of the non-specific phosphodiesterase inhibitor, pentoxifylline. This adds support to the argument that the inhibitory effects of hypertonic solution on methacholine-induced relaxation were, in part, related to a deficient generation of intracellular second messenger(s) required to produce comparable responses to those found in the isotonic solution. Furthermore, it is evident that the effects of hypertonic solution with sucrose did not appear to have an impact on the relaxant responses to sodium nitroprusside. Therefore, the process of relaxation per se was not inhibited by the hypertonic environment.

The effects of hypertonic solution on isoprenaline-mediated relaxation and its interrelationship with the generation of cyclic AMP is perhaps a little more complex. It is evident from the present investigation that an increase in the tonicity of Krebs solution does not affect the basal levels of cyclic AMP. In a previous study using single smooth muscle cells (A10), it was reported that sucrose (0.4 M) hypertonic solution did not affect basal levels of either cyclic AMP or inositol phosphate (Lutz and Kumar, 1993). However, it was reported that in hypertonic solution, vasopressin-stimulated production of cyclic AMP and inositol phosphate was significantly greater when compared to isotonic solution (Lutz and Kumar, 1993). In the present study, we find that although the vasorelaxant responses to isoprenaline are attenuated in hypertonic solution, the amount of cyclic AMP generated as result of the activation of β-adrenoceptors did not appear to be diminished by the increase in the osmolarity of Krebs solution. This finding may imply that additional mechanisms other than those associated with an elevation of cyclic AMP levels may contribute to the relaxant effects of isoprenaline in rat isolated aortic rings. There is evidence to suggest that in vascular smooth muscle, isoprenaline-mediated relaxation does not correlate well with the increase in the levels of cyclic AMP (Vegesna and Diamond, 1983, 1986). However, it has been suggested that if generation of cyclic AMP due to the activation of β-adrenoceptors is to account for relaxation in vascular smooth muscle, then some form of functional compartmentalization of cyclic AMP may exist (Vegesna and Diamond, 1983, 1986). Of course, an alternative explanation is that additional mechanisms other than an elevation of cyclic AMP levels in smooth muscle may be responsible for the vasorelaxant actions of isoprenaline.

Recently, it has become evident that under physiological conditions, the basal release of nitric oxide leading to activation of cyclic GMP results in a synergistic action with adenylate cyclase activators to produce relaxation in vascular smooth muscle. Evidence has been presented to indicate that reduced activity of nitric oxide synthase by either N^{ω}-nitro-L-arginine methyl ester or inhibition of soluble guanylate cyclase inhibitor by methylene blue could attenuate isoprenaline-mediated relaxation in rat isolated aortic rings (Delpy et al., 1996). In our current study, we find that the basal levels of cyclic GMP activity did not appear to be reduced. Therefore, it is unlikely that the reduced relaxation observed for isoprenaline in hypertonic solution was the results of reduced basal cyclic GMP action. This view is further supported by evidence that removal of the endothelium reduced the relaxation induced by forskolin (Delpy et al., 1996). In the present study, forskolin-mediated relaxations were not perturbed by an increase in the osmolarity of the Krebs solution.

An additional process that may participate in β-adrenoceptor-mediated relaxation is the K^{+}_{ATP}-sensitive channels. Activation of K^{+}_{ATP}-sensitive channels following the stimulation of β-adrenoceptors has been reported in coronary, mesenteric and pulmonary vascular smooth muscle (Katsuda et al., 1996; Huang and Kwok, 1997; Sheridan et al., 1997). This effects appears to be independent of the activity of cyclic AMP. However, the influence of hypertonic solution on isoprenaline-mediated relaxation is unlikely to be due to inhibition of the K^{+}_{ATP}-sensitive channels. In the present study, when we examined the influence of increased tonicity on pinacidil-induced relaxation, we found that its relaxations were not inhibited by an increase in the osmolarity of the Krebs solution. This would indicate that at least K^{+}_{ATP} channels sensitive to pinacidil did not participate in isoprenaline-mediated relaxation. Our findings seem to indicate that an additional mechanism, other than those described, may participate in isoprenaline-mediated relaxation in rat isolated aortic rings. This process appears to be sensitive to changes in osmolarity but it is not permanently disabled by changes in osmolarity. It is also apparent that in the presence of the phosphodiesterase inhibitor pentoxifylline, the inhibitory effects of hypertonic solutions could be reversed.

In healthy volunteers, intravenous infusion of hypertonic dextrose or sodium chloride solutions (1–4 mOsmol min^{-1}) has been found to increase forearm blood flow as a result of a decrease in resistance (Overbeck and Grega, 1970). In contrast, intravenous infusion of hyperosmolar sucrose, glucose or mannitol (1–2 Osmol) solutions into anaesthetized dogs has been reported to increase systemic arterial pressure, right atrial pressure and cardiac output (Stainsby and Barclay, 1971). Obviously, the impact of changes in osmotic pressure on overall haemodynamics in vivo is quite complex. It is evident that changes in the osmotic pressure of the extracellular fluid will not only have a direct effect on smooth muscle function but will

also affect neurogenic function of the muscle (McGrath and Shepherd, 1976). In addition, there most likely will be differences in the behavior of blood vessel function depending on the region.

In summary, we find that moderate changes in the osmolarity of the physiological salt solutions affects relaxant responses mediated via the β-adrenoceptor and muscarinic cholinoceptors in isolated blood vessel. An increase in osmolarity inhibited endothelium-dependent relaxation. This effect was associated with a reduced accumulation of the cyclonucleotide, cyclic GMP. However, an increase in the tonicity of Krebs solution did not affect basal levels of cyclic GMP, nor did it affect endothelium-independent relaxation induced by sodium nitroprusside. In contrast, the inhibitory effects of high osmolarity on β-adrenoceptor-mediated relaxation is neither due to a reduction in cyclic AMP generation, nor the result of inhibition of pinacidil-sensitive K^+_{ATP} channels. In addition, an increase in the tonicity of Krebs solution did not influence relaxation induced by direct activation of adenylate cyclase.

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