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Nitroglycerine- and isoprenaline-induced vasodilatation: assessment from the actions of cyclic nucleotides

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1 To investigate the vasodilator actions of nitroglycerine and isoprenaline, the effects of these agents, dibutyryl cyclic AMP (db cyclic AMP) and 8-bromo cyclic GMP (8-Br cyclic GMP) on intact muscle tissue, and of cyclic AMP and cyclic GMP on skinned muscle of the rabbit mesenteric artery were investigated.

2 In porcine coronary artery, nitroglycerine ($> 0.1\ \mu M$) increased the production of cyclic GMP with no change in the amount of cyclic AMP, while isoprenaline ($> 0.1\ \mu M$) significantly increased the production of cyclic AMP with no change in the amount of cyclic GMP.

3 In the rabbit mesenteric artery, nitroglycerine or isoprenaline inhibited the tonic component of the 39 mM $[K]_o$-induced contraction to a greater extent than the phasic component. Nitroglycerine and 8-Br cyclic GMP showed a stronger inhibitory action on the K-induced contraction than did isoprenaline and db cyclic AMP.

4 The sources of Ca utilized for the generation of contraction by noradrenaline and caffeine were estimated to be the same as those determined from the amplitudes of contractions evoked in Ca-free solution by various concentrations of noradrenaline or caffeine.

5 In intact muscle tissues, the effects of nitroglycerine or 8-Br cyclic GMP on the amount of Ca stored in cells were estimated from the caffeine-induced contraction in Ca-free solution. Both agents inhibited the contractions due to a reduction in the amount of Ca in the cells. When the effects of isoprenaline or db cyclic AMP were observed, both agents inhibited the caffeine-induced contraction but the accumulation of Ca into cells was greater than the control.

6 In saponin skinned muscles, the pCa-tension relationship in the presence of cyclic AMP and cyclic AMP-dependent protein kinase (cyclic AMP-PK) shifted to the right and to a lower level in comparison with the control. Applications of cyclic GMP with cyclic GMP-dependent protein kinase (cyclic GMP PK) also inhibited the contraction induced by low concentrations of Ca.

7 In skinned muscles, cyclic AMP exhibited dual actions on Ca store sites, i.e. in the presence of high concentrations of Ca or prolonged superfusion of Ca, cyclic AMP reduced the amount of Ca due to activation of the Ca-induced Ca release mechanism by excess accumulation of Ca. On the other hand, cyclic GMP consistently inhibited the amplitude of the caffeine-induced contraction due to a reduction in the amount of Ca in the store sites.

8 These results indicate that nitroglycerine and isoprenaline increase the amount of cyclic GMP and cyclic AMP, respectively. The main effect of cyclic GMP is activation of Ca extrusion, thus reducing the amount of Ca stored in the cell, while the main effect of cyclic AMP is to increase the amount of Ca stored in the cell. Both cyclic AMP with cyclic AMP-PK and cyclic GMP with cyclic GMP-PK inhibit the phosphorylation of myosin. Consequently both cyclic nucleotides reduce the free Ca in the myoplasm and promote relaxation, but by different mechanisms.

Introduction

Nitroglycerine does not affect the membrane potential and resistance in either smooth muscle cells of the pig, dog and rabbit coronary arteries, or in those of the guinea-pig and rabbit mesenteric arteries. Furthermore, this agent does not modify neuromuscular transmission in the pig and guinea-pig mesenteric

arteries. However, nitroglycerine consistently inhibits the contractions evoked by displacement of the membrane potential, action potentials, and by excess concentrations of $[K]_o$, noradrenaline or acetylcholine in the above mesenteric and coronary arteries. Nitroglycerine does not inhibit the phosphorylation of myosin for triggering the contraction, or the release of Ca from store sites in skinned muscle tissues, but it does reduce the amount of stored Ca. Also, it accelerates the Ca efflux from single cell pellets in the presence of stimulant agonists (Ito *et al.*, 1980a,b; 1981a,b; Itoh *et al.*, 1983b).

Organic nitrate compounds including nitroglycerine, isosorbite dinitrate, nicorandil and sodium nitroprusside increase the amount of cyclic GMP produced in various vascular smooth muscle tissues (Schultz *et al.*, 1977; Kukovetz *et al.*, 1979; Axelsson *et al.*, 1981; Gruetter *et al.*, 1981; Endoh & Taira, 1983). On the other hand, isoprenaline activates β-adrenoceptors and produces cyclic AMP. Administration of cyclic AMP- and cyclic AMP-dependent protein kinase (cyclic AMP-PK; A-kinase) inhibits phosphorylation of myosin (Adelstein & Eisenberg, 1980; Itoh *et al.*, 1982), and increases Ca uptake into the Ca storage site (microsomal fractions) (Webb & Bhalla, 1976; Nishikori *et al.*, 1977; Bhalla *et al.*, 1978; Thorens & Haeusler, 1978; Brockbank & England, 1980). Furthermore, cyclic AMP possesses the ability to activate the Ca-induced Ca release mechanism due to excess accumulation of Ca into the storage site (Itoh *et al.*, 1981b; 1982), and it activates the Ca-pump at the sarcolemma (Gopinath & Vincenzi, 1977; Caroni & Carafoli, 1981; Colca *et al.*, 1983).

If the action of nitroglycerine or isoprenaline is dependent on the production of cyclic GMP or cyclic AMP, nitroglycerine and 8-bromo cyclic GMP (8-Br cyclic GMP) or isoprenaline and dibutyryl cyclic AMP (db cyclic AMP) may have the same mechanism of vasodilatation. In this paper we have attempted to clarify the mechanism of nitroglycerine-induced vasodilatation, in relation to the synthesis and functions of cyclic GMP. For investigations on the intact muscle tissue, 8-Br cyclic GMP was used, and for skinned muscles, cyclic GMP was used. Actions of isoprenaline, cyclic AMP and db cyclic AMP were also studied and compared to those of nitroglycerine, cyclic GMP and 8-Br cyclic GMP.

Methods

Albino rabbits of either sex (2–3 kg) were given sodium pentobarbitone 40 mg kg^{-1} i.v. and exsanguinated. The mesentery in the jejunal region was immediately placed in a dissecting chamber filled with Krebs solution. The mesenteric artery was carefully excised under a binocular microscope.

The composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.0, glucose 11.5. It was aerated with 97% O_2 plus 3% CO_2 and the pH was adjusted to 7.3. High-$[K]_o$ solution was prepared by replacing NaCl with KCl, isosmotically.

Recordings of mechanical activity

Mechanical responses were measured by attaching a circular strip of the mesenteric artery (0.05–0.1 mm in width and 0.3 mm in length) to a strain gauge (U-gauge, Shinko, Tokyo). The tissue was superfused in an organ bath (0.9 ml) filled with Krebs solution. Solutions containing drugs of modified ionic concentrations were added to the bath during simultaneous water pump aspiration of the solution already present. The test solutions could be added in only a few seconds.

Skinned tissues were obtained using saponin according to the method described elsewhere (Itoh *et al.*, 1981a,b). After a K-induced contraction of an intact muscle had been recorded, the bathing solution was replaced with a relaxing solution containing 130 mM K-methanesulphonate (KMs), 20 mM Tris maleate, 5 mM $Mg(Ms)_2$, 5 mM ATP (10 mM Na as Na_2 ATP) and 4 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N,',N'-tetra acetic acid (EGTA) at pH 6.8. The tissue was left for 20 min in the relaxing solution containing 25 μg ml^{-1} saponin and was washed again with the same solution and left until the tension became constant at about zero level. Immediately before the addition of a Ca-containing solution, the preparation was again superfused with the relaxing solution. To investigate the effects of various drugs on the Ca receptor of contractile proteins, the pCa-tension curve was observed in the presence or absence of drugs. Various Ca concentrations were prepared by adding appropriate amounts of $Ca(Ms)_2$ to EGTA. The apparent binding constant of EGTA for Ca was taken to be 10^6 mol^{-1} at pH 6.8 at 25°C (Itoh *et al.*, 1981a). The pH of the relaxing and various Ca solutions was kept at 6.8 by isosmotically replacing the appropriate amount of KMs with KOH.

Assay of cyclic AMP and cyclic GMP

Smooth muscle tissues excised from porcine coronary arteries were prepared in cold Krebs solution, under a binocular microscope. Dissected tissues, weighing 50–100 mg wet wt. (80 mg in most preparations), were mounted in an organ bath without any load. Tissue kept at 37°C for 60 min in the aerated Krebs solution served as the control. To observe the effects of isoprenaline or nitroglycerine on the cyclic AMP or cyclic GMP content, various concentrations of these agents were added for the final 3 min of the 60 min incubation period (Itoh *et al.*, 1982).

After 60 min incubation, the tissue was frozen with nitrogen and homogenized in 6% trichloroacetic acid. The levels of cyclic AMP and cyclic GMP in the extracts were measured using a radioimmunoassay kit (Yamasa Shoyu: Honma *et al.*, 1977).

Drugs

Chemicals used in the present experiments were caffeine (NE Wako Pure Chemical Industries, Osaka, Japan), saponin (ICN Pharmaceuticals. Inc., Cleveland, Ohio), noradrenaline HCl (Sigma Chemical Co., St. Louis, MO), isoprenaline (Tokyo Kasei Kogyo Co., Tokyo, Japan), prazosin (Pfizer Pharmaceutical Co., Basel, Switzerland), guanethidine (Tokyo Kasei Kogyo Co.), nitroglycerine (water soluble type: Nihon-Kayaku Co., Tokyo, Japan), dibutyryl cyclic AMP (db cyclic AMP; Sigma), 8-bromo cyclic GMP (8-Br cyclic GMP; Sigma), cyclic AMP-dependent protein kinase (cyclic AMP-PK; P5511, Sigma) and ethyleneglycol-bis- (β-aminoethylether) N,N,N',N'- tetra acetic acid (EGTA; Dozin Laboratories, Kumamoto, Japan). Cyclic GMP-dependent protein kinase (cyclic GMP-PK; Nakasawa & Sano, 1975) was kindly provided by Prof. H. Hidaka and Dr M. Inagaki, Mie University, Japan.

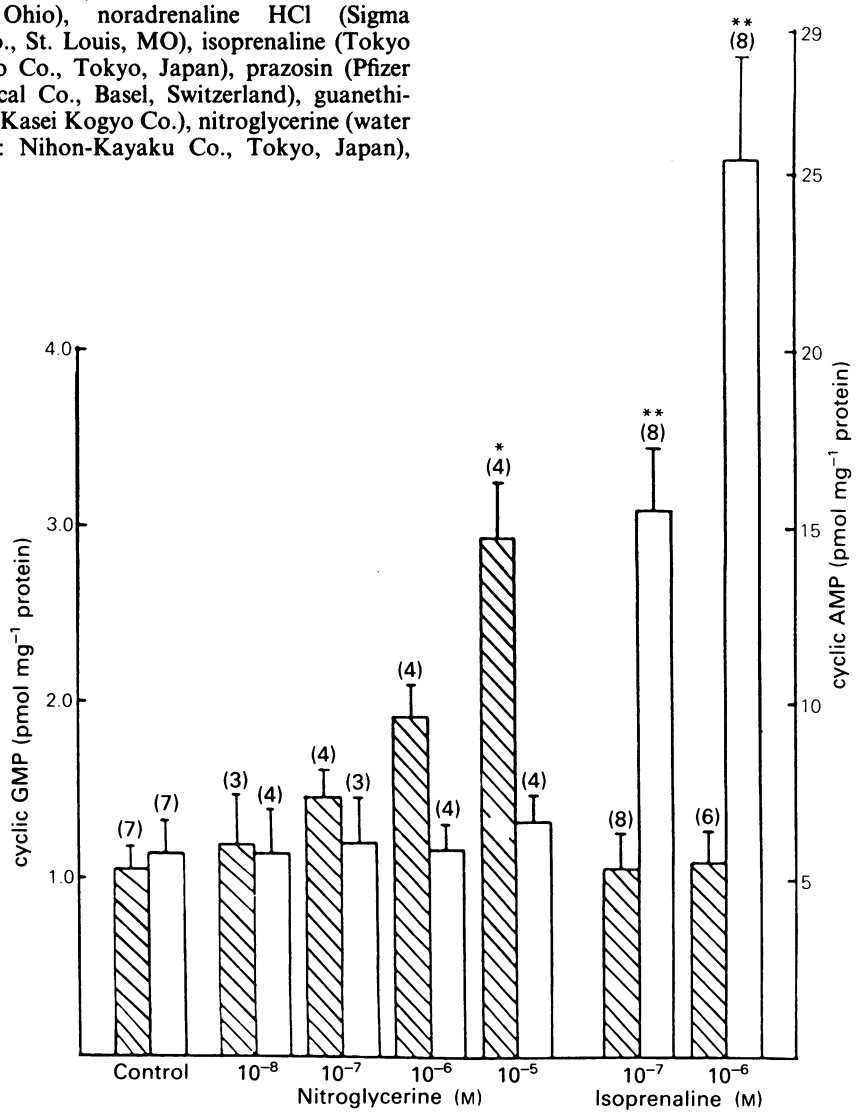

Figure 1 Effects of nitroglycerine and isoprenaline on the production of cyclic AMP (open columns) and cyclic GMP (hatched columns) in the porcine coronary artery. Each column represents the mean of a number of preparations (shown by number in parentheses) and vertical lines show s.d. Significantly different from the control, $*P < 0.05$; $**P < 0.01$.

Statistics

The results were expressed as the mean value ± s.d. (n = number of observations), and statistical significance was assessed using either Student's t test for paired observations or that for unpaired observations between two drugs. P values of less than 0.05 were considered to be significant.

Results

Effects of isoprenaline and nitroglycerine on the production of cyclic AMP and cyclic GMP

Figure 1 shows the effects of various concentrations of isoprenaline or nitroglycerine on the production of cyclic AMP and cyclic GMP in the porcine coronary

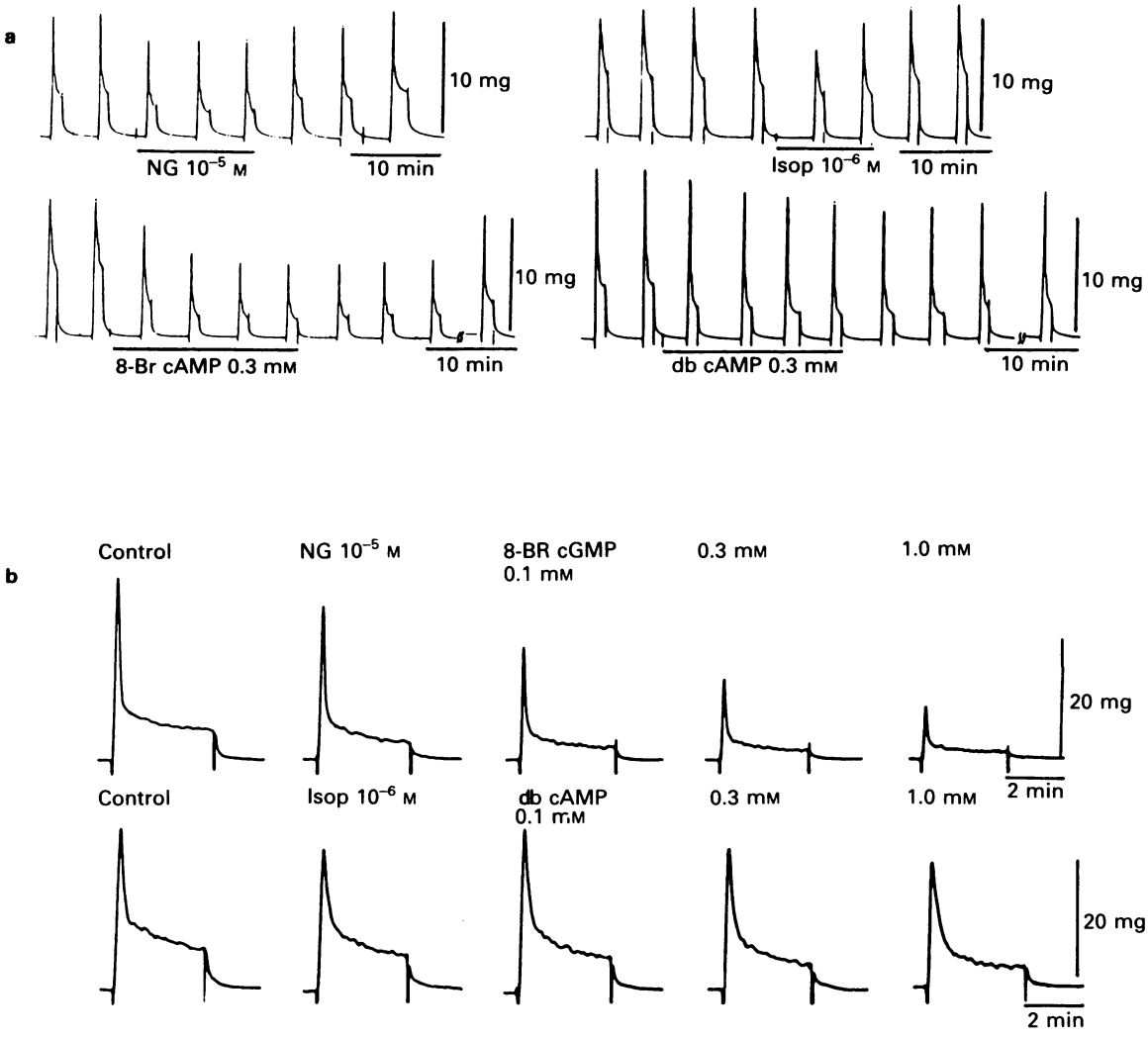

Figure 2 Effects of nitroglycerine (NG), 8-bromo cyclic GMP (8-Br cGMP), isoprenaline (Isop) and dibutyryl cyclic AMP (db cAMP) on contractions induced by 39 mM $[K]_o$ in the rabbit mesenteric artery. Guanethidine (1 μM) and prazosin (1 μM) were present throughout experiments to prevent the release of noradrenaline from nerve terminals and the activation of α-adrenoceptors, respectively. (a) Effects of the above agents on K-induced contractions (2 min administration of 39 mM $[K]_o$ every 5 min). (b) Effects of various agents on the shape of the K-induced contractions (39 mM $[K]_o$). The agents were administered 3 min before the addition of 39 mM $[K]_o$.

artery after 3 min incubation. In comparison to the control, nitroglycerine consistently increased the amount of cyclic GMP in concentrations greater than 0.1 μM ($P < 0.05$) with no change in the production of cyclic AMP ($P > 0.05$). Administration of isoprenaline (more than 0.1 μM) markedly increased the concentration of cyclic AMP ($P < 0.01$) with no change in the level of cyclic GMP ($P > 0.05$). these results indicate that nitroglycerine and isoprenaline selectively increased the concentrations of cyclic GMP and cyclic AMP, respectively.

Effects of nitroglycerine, isoprenaline and cyclic nucleotides on the K-induced contraction

Figure 2a shows the effects of nitroglycerine, isoprenaline, 8-Br cyclic GMP and db cyclic AMP on the 39 mM [$K[_o$-induced contraction in rabbit mesenteric artery. Excess concentrations of $[K]_o$ were successively added for 2 min every 5 min. Guanethidine (1 μM) was present in the solution throughout the experiment to prevent the effect of noradrenaline released from nerve terminals by depolarization. When high concentrations of isoprenaline were tested against the K-induced contraction, 1 μM prazosin was present to prevent the activation of α-adrenoceptors. Nitroglycerine (10 μM) and isoprenaline (1 μM) reduced the amplitude of the K-induced contraction within a few minutes. However, with 8-Br cyclic GMP and db cyclic AMP more than 10 min was required to give a steady level of inhibition of the contraction. The K-induced contraction consisted of phasic and tonic components (Figure 2b). Following 5 min superfusion with nitroglycerine, isoprenaline, 8-Br cyclic GMP or db cyclic AMP, 39 mM $[K]_o$ was administerd. When either 8-Br cyclic GMP or db cyclic AMP, in concentrations over 0.1 mM, was administered, both phasic and tonic components of the K-induced contraction were inhibited dose-dependently. However, the inhibition induced by db cyclic AMP was less than that seen with 8-Br cyclic GMP, i.e. a 10 times higher concentration of db cyclic AMP was required to give the same extent of inhibition as that observed with 8-Br cyclic GMP (0.1 mM cyclic GMP vs 1 mM db cyclic AMP in Figure 2).

Effects of caffeine or noradrenaline on Ca storage in smooth muscle cells of the mesenteric artery

The following experiments were done to assess the effects of nitroglycerine, isoprenaline or cyclic nucleotides on the Ca storage site used to elicit the caffeine- or noradrenaline-induced contractions evoked in the Ca-free 2 mM EGTA-containing solution. After depletion of Ca from the store by repeated administration of 20 mM caffeine in Ca-free solution, 2.5 mM Ca was added for 20 min (1st procedure), the tissue was then superfused with Ca-free 2 mM EGTA-containing solution for 10 min (2nd procedure), and subsequently, various concentrations of caffeine were administered as the conditioning stimulus (3rd procedure). Finally after 5 min superfusion with Ca-free solution, 20 mM caffeine was again added in Ca-free solution as the test stimulus (4th procedure). With the addition of 1 mM caffeine as the conditioning stimulus, a contraction was evoked, and 20 mM caffeine subsequently administered as the test stimulus produced a small contraction (Figure 3a1). Increased concentrations of caffeine administered as the conditioning stimulus proportionally reduced the amplitude of the contraction subsequently evoked by the test administration of caffeine but enhanced, in a dose-dependent manner, the amplitude and the rates of rise and fall of the contraction (Figure 3b). Figure 3c shows the responses of smooth muscle cells to caffeine (20 mM) given as the test stimulus following conditioning treatments with various concentrations of caffeine. When concentrations of caffeine given as the conditioning stimulus were increased to 5 mM or beyond, the test stimulus did not produce a contraction.

When 0.1 μM noradrenaline was the conditioning stimulus, a small contraction was evoked. Increased concentrations of noradrenaline enlarged the contraction dose-dependently. As a consequence, the amplitude of the resulting caffeine-induced contraction, evoked by the test stimulus, was proportionally reduced (Figure 3d). These results indicate that noradrenaline and caffeine release Ca from the same storage site in smooth muscle cells. The noradrenaline-induced contraction evoked in Ca-free solution ceased with the addition of prazosin (1 μM). The shape of the contractions evoked by caffeine or noradrenaline as the conditioning stimulus were compared (Figure 3e). The rates of rise and fall of the contractions were faster for the caffeine-induced contraction than those observed for the noradrenaline-induced contraction.

Effects of nitroglycerine, isoprenaline or cyclic nucleotides on Ca storage in intact muscle cells

The effects of nitroglycerine, isoprenaline or cyclic nucleotides on the caffeine-induced contraction evoked in Ca-free 2 mM EGTA-containing solution were observed. When 10 μM nitroglycerine with 2.5 mM Ca was applied for 20 min during the 1st procedure, the amplitude of the caffeine-induced contraction evoked by the conditioning stimulation was reduced (0.81 ± 0.12 times the control, $n = 5$; $P < 0.05$). When 1 μM isoprenaline plus 2.5 mM Ca was administered, the amplitude of the caffeine-induced contraction was slightly enlarged (1.12 ± 0.8 times the control, $n = 5$; $P < 0.05$). On the addition of 8-Br cyclic GMP (0.1 mM) with Ca, the amplitude of the contraction was reduced (0.75 ± 0.14 times, $n = 5$;

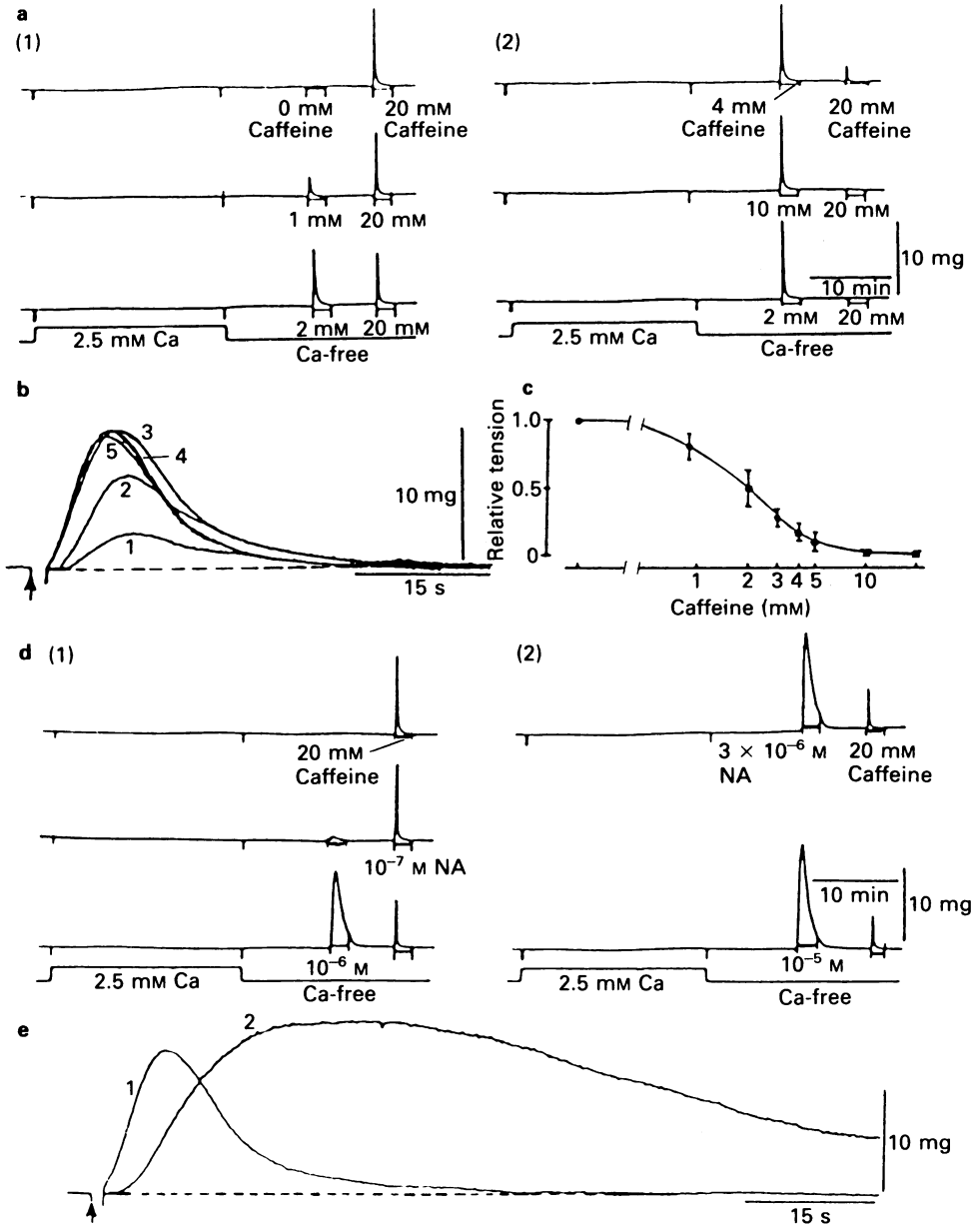

Figure 3 (a) Responses of smooth muscle cells of the rabbit mesenteric artery to caffeine in Ca-free solution containing 2 mM EGTA. After depletion of stored Ca by repeated administration of caffeine (20 mM), 2.5 mM Ca was given for 20 min (1st procedure), then the tissue was rinsed with Ca-free solution containing 2 mM EGTA for 10 min (2nd procedure) and various concentrations of caffeine were subsequently administered (1 mM – 20 mM) for 2 min (3rd procedure; conditioning stimulus). After the addition of various concentrations of caffeine in Ca-free solution, 20 mM caffeine was again added (4th procedure; test stimulus). (b) Traces of contractions evoked by various concentrations of caffeine (as the conditioning stimulus). (1) 1 mM, (2) 2 mM, (3) 4 mM, (4) 10 mM and (5) 20 mM caffeine. (c) Amplitudes of contraction evoked by 20 mM caffeine (as the test stimulus) following treatment with various concentrations of caffeine (conditioning stimulus). Horizontal scale indicates the concentration of caffeine administered during the conditioning stimulation and vertical scale indicates the amplitude of the contraction evoked by the test stimulation with caffeine. The amplitude of contraction evoked by the test stimulation without the addition of a conditioning stimulus was normalized as 1.0. Vertical lines indicate s.d., $n = 4-6$. (d) Responses of smooth muscle cells of the rabbit mesenteric artery to noradrenaline (NA; conditioning stimulus) and to subsequently administered caffeine (test stimulus; 20 mM) in Ca-free solution containing 2 mM EGTA. Concentration of NA varied from 0.1 M to 10 μM. (e) Actual traces of the mechanical response evoked by 10 μM noradrenaline (1) or 20 mM caffeine (2) as conditioning stimuli. Two records obtained in successive experiments were superimposed.

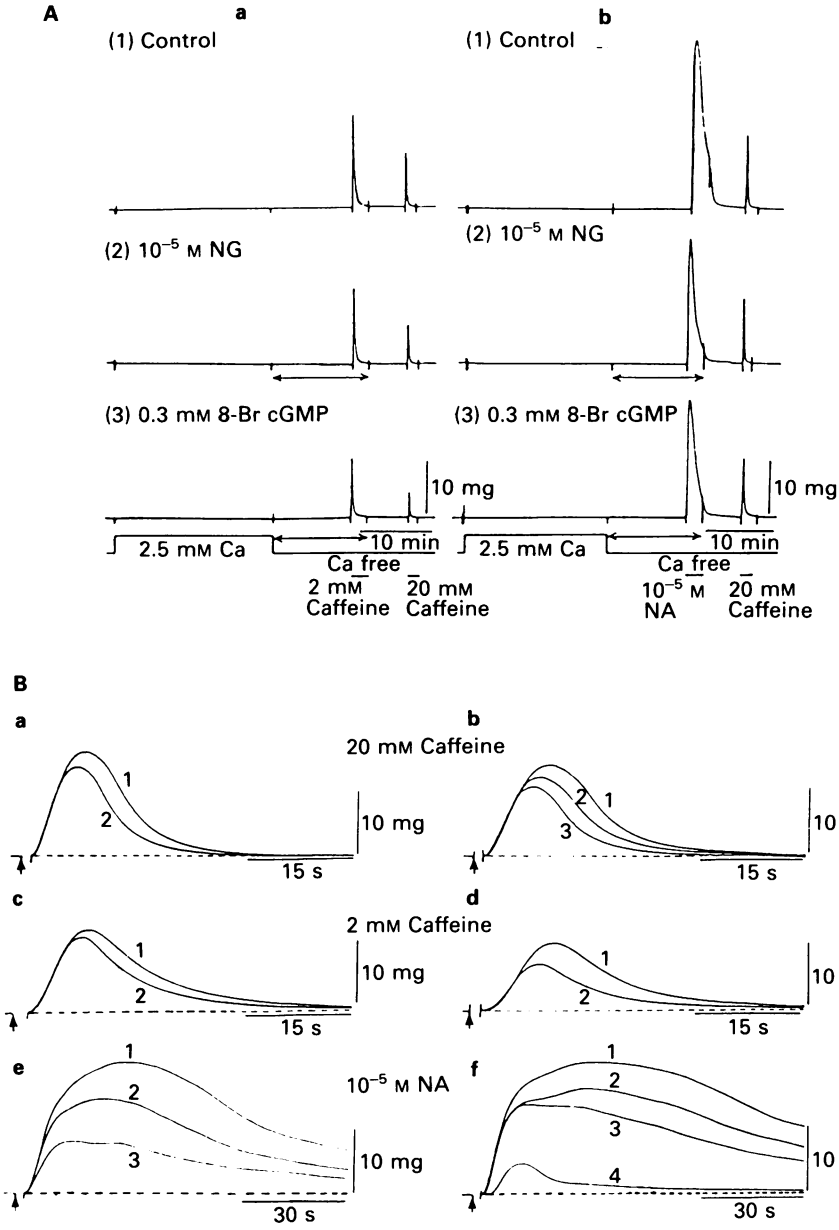

Figure 4 (A) Effects of nitroglycerine (NG; 10^{-5} M or 8-bromo cyclic GMP (8-Br cGMP; 0.3 mM) on caffeine- or noradrenaline (NA)-induced contractions in Ca-free solution containing 2 mM EGTA (rabbit mesenteric artery). Experimental procedures were the same as those described in Figure 3a. Nitroglycerine or 8-bromo cyclic GMP were added during the 2nd and 3rd procedures. As the conditioning stimulus, either 2 mM caffeine (a) or 10 μM noradrenaline (b) was applied and as the test stimulus 20 mM caffeine was applied (a and b). (B) Changes in the shape of the contraction evoked by caffeine, 20 mM (a,b) and 2 mM (c,d), or noradrenaline, 10^{-5} M (e,f), with nitroglycerine or 8-bromo cyclic GMP present during procedures 2–3 (see text and legend to Figure 3). Experimental procedures were the same as those described in (A). Intervals between drug additions were 40 min. Contractions evoked before and during the administration of the above agents were superimposed. (a) Control (1), + nitroglycerine 10^{-5} M (2); (b) control (1), + 8-Br cyclic GMP 0.3 mM (2), 1 mM (3); (c) control (1), + nitroglycerine 10^{-5} M (2); (d) control (1), + 8-Br cyclic GMP 0.3 mM (2); (e) control (1), + nitroglycerine 10^{-5} M (2), 2×10^{-5} M (3); (f) control (1), + 8-Br cyclic GMP 0.1 mM (2), 0.3 mM (3), 1 mM (4). Dotted lines indicate the level of resting tension.

$P < 0.05$ compared to the control; $P > 0.1$, compared to that observed in the presence of nitroglycerine), but db cyclic AMP (0.1 mM) enhanced the amplitude (1.21 ± 0.11 times, $n = 5$; $P < 0.02$, compared to the control; $P > 0.1$, compared to that observed in the presence of isoprenaline).

With the addition of 10 μM nitroglycerine during the 2nd and 3rd procedures, the resulting caffeine-induced contractions evoked by the conditioning and test stimulations were reduced (Figure 4A a1 vs a2; conditioning and test responses were 0.81 ± 0.10 times the control ($PL < 0.01$) and 0.68 ± 0.16 times the control ($P < 0.01$, respectively). The contractions evoked by conditioning and test stimulations were both normalized as 1.0. Much the same response was observed on the addition of 0.3 mM 8-Br cyclic GMP (conditioning responses: 0.74 ± 0.19 times the control, $P < 0.05$ and $P > 0.1$, compared to that observed in the presence of nitroglycerine; test response: 0.39 ± 0.8 times the control, $P < 0.001$ and $P < 0.05$, compared to that observed in the presence of nitroglycerine. Figure 4Aa3). With the addition of 10 μM noradrenaline as the conditioning stimulus, the amplitude of the caffeine-induced (test) contraction was consistently reduced by pretreatment with 10 μM nitroglycerine or 0.3 mM 8-Br cyclic GMP during the 2nd and 3rd procedures (conditioning and test responses; 0.81 ± 0.10 times ($P < 0.02$) and 0.77 ± 0.14 times the control ($P < 0.05$), respectively, $n = 5$; Figure 4Ab3).

Figure 4B shows the effects of nitroglycerine or 8-Br cyclic GMP on the shapes of the contraction evoked by caffeine or noradrenaline administered as the conditioning stimulus in the Ca-free solution. When either nitroglycerine (10 μM) or 8-Br cyclic GMP (0.3–1.0 M) was added during the 2nd and 3rd procedures, the peak amplitudes of both the 20 mM and 2 mM caffeine-induced contractions were reduced with no change in the rising phase of contractions. As a consequence, the time to reach the peak amplitude and the duration of contraction were reduced (Figure 4B

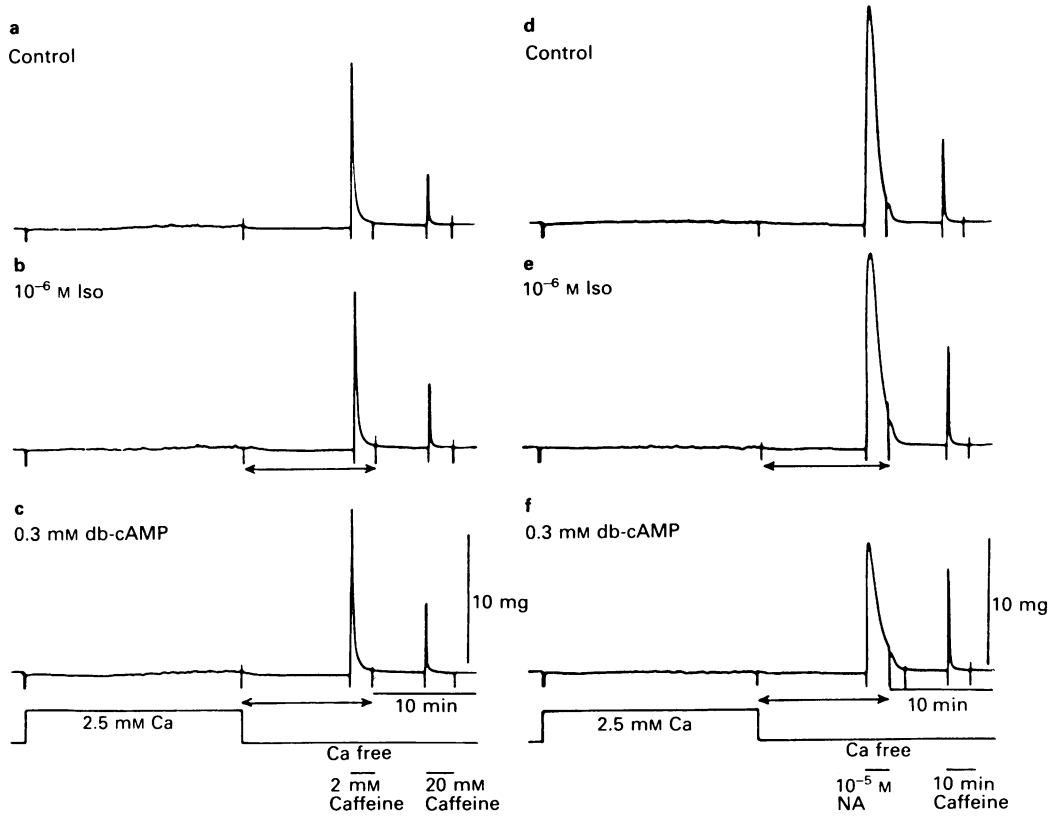

Figure 5 Effects of isoprenaline or dibutyryl cyclic AMP on the caffeine- or noradrenaline (NA)-induced contraction in Ca-free solution containing 2 mM EGTA. Experimental procedures were the same as those described in Figure 3a. Isoprenaline (Iso) or dibutyryl cyclic AMP (db-cAMP) were added during the 2nd and 3rd procedures. As the conditioning stimulus 2 mM caffeine (a–c) or 10 μM noradrenaline (d–f) was given and as the test stimulus 20 mM caffeine was applied.

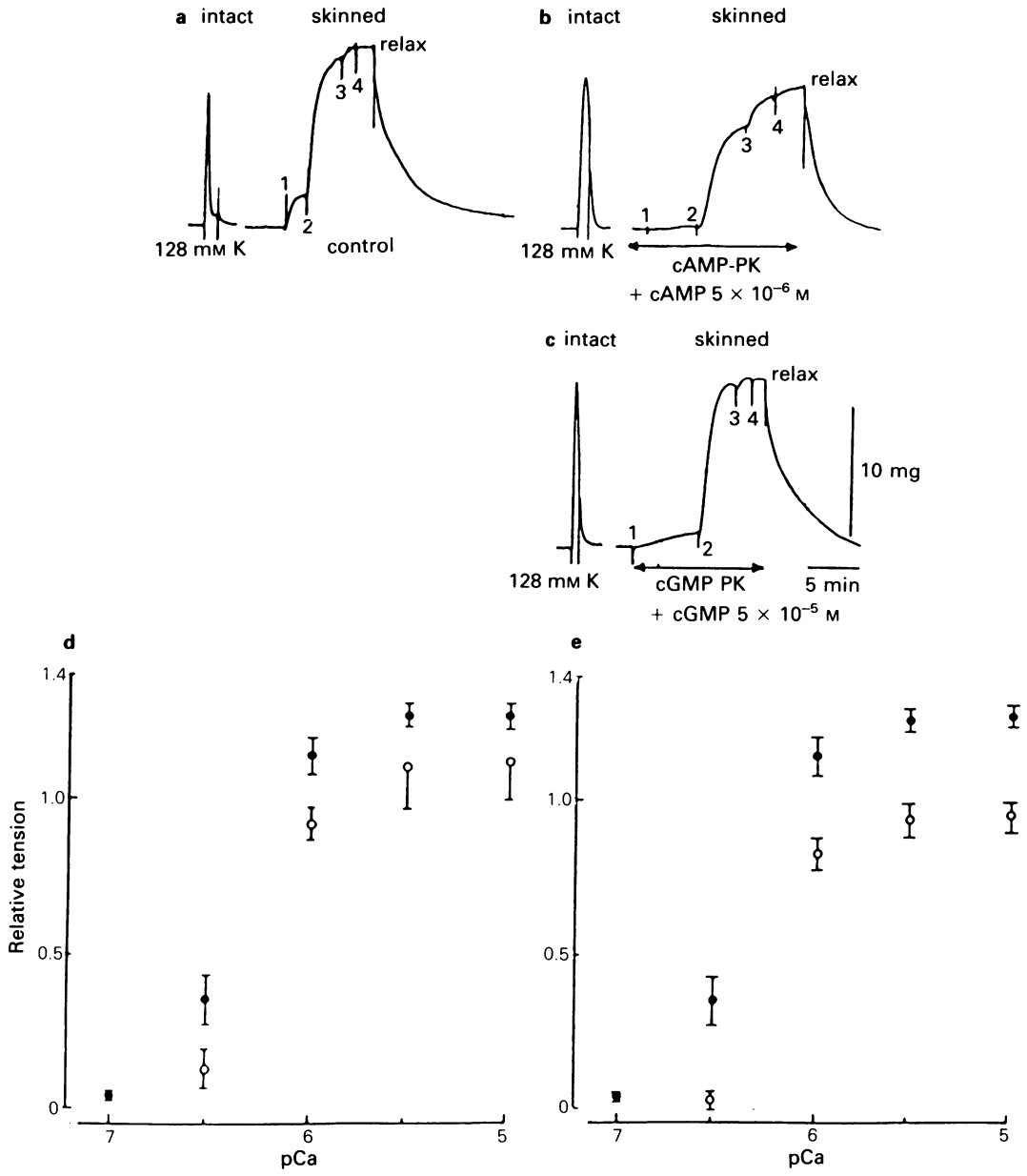

Figure 6 Effects of cyclic AMP with cyclic AMP-dependent protein kinase (cAMP-PK) or cyclic GMP with cyclic GMP-dependent protein kinase (cGMP-PK) on the Ca-induced contraction in skinned muscles tissues excised from the rabbit mesenteric artery. (a) Control; before skinning the tissue, 128 mM K was added. After skinning with 25 $\mu g\,ml^{-1}$ saponin for 20 min, concentrations of Ca from 0.3 μM were added cumulatively; (1) 0.3 μM Ca, (2) 1 μM Ca, (3) 3 μM (4) 10 μM Ca. (b) Effects of 50 μM cyclic AMP with 50 $\mu g\,ml^{-1}$ cyclic AMP-PK on the Ca-induced contraction. (c) Effects of 50 μM cyclic GMP with 1.0 $\mu g\,ml^{-1}$ cyclic GMP-PK on the Ca-induced contraction. Relaxation; the tissue was rinsed with the relaxing solution (ionic composition described in Methods). (d and e) Effects of cyclic AMP with cyclic AMP-PK or cyclic GMP with cyclic GMP-PK on the pCa-tension relationship observed in skinned muscle tissues of the rabbit mesenteric artery. (d) Effects of 1 μM cyclic GMP with 1.0 $\mu g\,ml^{-1}$ cyclic GMP-PK (○) on the Ca-induced contraction; (●) control. (e) Effects of 50 μM cyclic AMP with 50 $\mu g\,ml^{-1}$ cyclic AMP (○) on the Ca-induced contraction; (●) control. Experimental procedures were the same as those described above (a–c). The amplitude of the 128 mM $[K]_o$-induced contractions was normalized as 1.0, and amplitudes of Ca-induced contractions evoked in skinned muscles were compared in the presence or absence of cyclic nucleotides. Each point shows the mean and vertical lines indicate s.d. ($n = 3-6$).

a–d). When 10 μM or 20 μM nitroglycerine or 0.1 mM–1 mM 8-Br cyclic GMP were tested against the 10 μM noradrenaline-induced contraction, the peak amplitude and the rate of fall of the 10 μM noradrenaline-induced contraction and the time required to reach the peak amplitude were also reduced (e and f).

Using the same experimental procedures, either 1 μM isoprenaline or 0.3 mM db cyclic AMP was administered during the 2nd and 3rd procedures. With the administration of 2 mM caffeine as the conditioning stimulus, amplitudes of contractions were slightly reduced (0.84 ± 0.08 times the control for isoprenaline, $P < 0.02$; and 0.91 ± 0.05 times the control for db cyclic AMP, $P < 0.02$; $n = 5$), but the subsequently applied test stimulus (20 mM caffeine) gave a greater contraction (Figure 5,a vs b or c; 1.24 ± 0.13 times the control for isoprenaline, $P < 0.02$; and 1.35 ± 0.14 times for db cyclic AMP, $P < 0.01$; $n = 5$). Isoprenaline (1 μM) or db cyclic AMP (0.3 mM) added during the 2nd and 3rd procedures reduced the amplitude of the contraction evoked when 10 μM noradrenaline was used as the conditioning stimulus (0.84 ± 0.18 times for isoprenaline, $P < 0.05$; and 0.64 ± 0.20 times the control for db cyclic AMP, $P < 0.05$; $n = 5$). However, the resulting contraction evoked by the test stimulus (20 mM caffeine) was enhanced in comparison to that observed in the control (1.38 ± 0.14 times after isoprenaline and 1.51 ± 0.16 times after db cyclic AMP, $P < 0.01$; $n = 5$ (d vs e or f).

Effects of cyclic AMP and cyclic GMP on contractile proteins and accumulation of Ca into storage sites in skinned muscles

Before the addition of saponin 25 μg ml^{-1} for 20 min, 128 mM $[K]_o$ ws applied to evoke a contraction. After skinning, various concentrations of free Ca, ranging between 0.3 μM and 10 μM were applied cumulatively (Figure 6A a–c). In the presence of 0.3 μM Ca, a contraction was evoked and 3 μM Ca produced a larger contraction than that observed after the addition of 128 mM $[K]_o$ to the intact tissue (a). When 50 μM cyclic AMP with 50 μg ml^{-1} cyclic AMP-PK were present throughout the experiments, the Ca-induced contraction was markedly inhibited, i.e. 0.3 μM Ca did not produce a contraction and the amplitude of the contraction induced by 3 μM Ca was smaller than that evoked by 128 mM $[K]_o$ (b). When 50 μM cyclic GMP with 1 μg ml^{-1} cyclic GMP-PK were applied to the tissue, the amplitude of the contraction induced by 0.3 μM Ca was slightly reduced, but that induced by 1 μM Ca was inhibited to a lesser extent than that observed following the administration of cyclic AMP with cyclic AMP-PK (c).

Figure 6 (d and e) shows the pCa-tension relationship observed in the presence of cyclic GMP (with

Table 1 Effects of cyclic AMP (10^{-6} M) or cyclic GMP (10^{-6} M) in the absence or presence of cyclic AMP- and cyclic GMP-dependent protein kinases (50 μg ml^{-1} and 10 μg ml^{-1}, respectively) on the Ca-induced contractions of skinned muscle tissues excised from rabbit mesenteric artery (A) and the effects on the accumulation of Ca into the store site (B)

A	*Control*	*cyclic AMP*	*cyclic AMP cyclic AMP-PK*	*cyclic GMP*	*cyclic GMP cyclic AMP-PK*	*cyclic GMP cyclic GMP-PK*
10^{-6} M Ca-induced contraction	100	99.1 ± 1.5	82.5 ± 11.5*	95.2 ± 4.3	85.3 ± 6.8*	80.2 ± 9.1*

B	*Control*	*cyclic AMP*		*cyclic GMP*	
		10^{-7} M	10^{-6} M	10^{-7} M	10^{-6} M
10^{-6} M Ca for 30 s	100	117.3 ± 5.8**	113.3 ± 5.2**	83.4 ± 6.1**	67.5 ± 12.5**
10^{-6} M Ca for 120 s	100	98.6 ± 6.8	71.7 ± 10.6**	none	73.8 ± 10.4*

(B) Cyclic nucleotide was added for 60 s before and during the administration of 1 μM Ca for either 30 s or 120 s and the amount of stored Ca was estimated from the amplitude of the caffeine-induced contraction. The amplitude of the caffeine-contraction in the absence of cyclic nucleotide was normalized as 100%. Experimental procedures were the same as those described in Figure 7. Results show mean % change ± s.d.; $n = 3–5$ (A) and $n = 3–7$ (B). *$P < 0.05$, **$P < 0.01$, compared to the control value.

$1\,\mu g\,ml^{-1}$ cyclic GMP-PK) or cyclic AMP (with $50\,\mu g\,ml^{-1}$ cyclic AMP-PK). The amplitude of the contraction evoked by 128 mM K in the intact tissue was normalized as 1.0. Administration of 1 μM cyclic GMP with $1\,\mu g\,ml^{-1}$ cyclic GMP-PK reduced the amplitude of the contraction evoked by Ca at concentrations below 1 μM ($P < 0.01$, compared to the control; Figure 6d). When 50 μM cyclic AMP and $50\,\mu g\,ml^{-1}$ cyclic AMP-PK were applied simultaneously, the contractions to concentrations of Ca ranging between 0.1 μM and 10 μM were consistently inhibited ($P < 0.01$ compared to the control; Figure 6e).

To observe the effects of cyclic AMP or cyclic GMP on the accumulation of Ca into the storage site, the amount of Ca stored in the cell was estimated from the amplitude of the caffeine-induced contraction following treatment of skinned muscles with Ca. After skinning the tissue, 1 μM Ca was added for 30 s and the tissue was then rinsed for 3 min with the relaxing solution containing 0.1 mM EGTA. Subsequently, 20 mM caffeine was added so that the amount of Ca could be estimated from the amplitude of the contraction. When cyclic AMP 0.1 μM was administered before and during the addition of 1 μM Ca for 1 min, the resulting caffeine-induced contraction was slightly enlarged (Figure 7a), whereas, the amplitude of the caffeine-contraction was reduced in the presence of cyclic GMP 0.1 μM (Figure 7d).

Table 1 summarizes the effects of cyclic AMP and cyclic GMP on the Ca- and caffeine-induced contractions in skinned muscles. Following the addition of 1 μM Ca for 2 min, the amplitude of the contraction was taken as 100%. When either 1 μM cyclic AMP or cyclic GMP was administered before and during the addition of Ca for 1 min, the resulting contractions induced by Ca 1 μM were $99.1 \pm 1.5\%$ ($P > 0.1$, $n = 5$) or $95.2 \pm 4.3\%$ of the control ($P = 0.05$, $n = 3$), respectively. With the addition of 1 μM cyclic AMP and cyclic AMP-PK ($50\,\mu g\,ml^{-1}$), the contraction to Ca was reduced and with the addition of cyclic GMP and cyclic GMP-PK ($1.0\,\mu g\,ml^{-1}$) it was reduced to a value smaller than the contraction observed after cyclic GMP alone (Table 1). These results indicate that cyclic nucleotides have only a slight effect on the Ca-induced contraction in the absence of protein kinases (Table 1A). The effects of cyclic AMP or cyclic GMP on Ca accumulation were, therefore, estimated from the amplitude of caffeine-contraction in the absence of cyclic AMP-PK or cyclic GMP-PK. As shown in Table 1B, cyclic AMP (0.1 μM and 1 μM) increased the amplitude of the caffeine-induced contraction after a 30 s incubation with Ca, whereas after a 120 s incubation with Ca, 0.1 μM cyclic AMP did not, but 1 μM cyclic AMP did, reduce the amplitude of the caffeine-induced contraction. On the other hand, cyclic GMP (0.1 μM and 1 μM; 30 and 120 s Ca incubation) consistently inhibited the amplitude of the caffeine-induced contraction.

These results indicate that cyclic AMP increases the accumulation of Ca into the storage site, yet when the amounts of Ca in the store are increased by longer

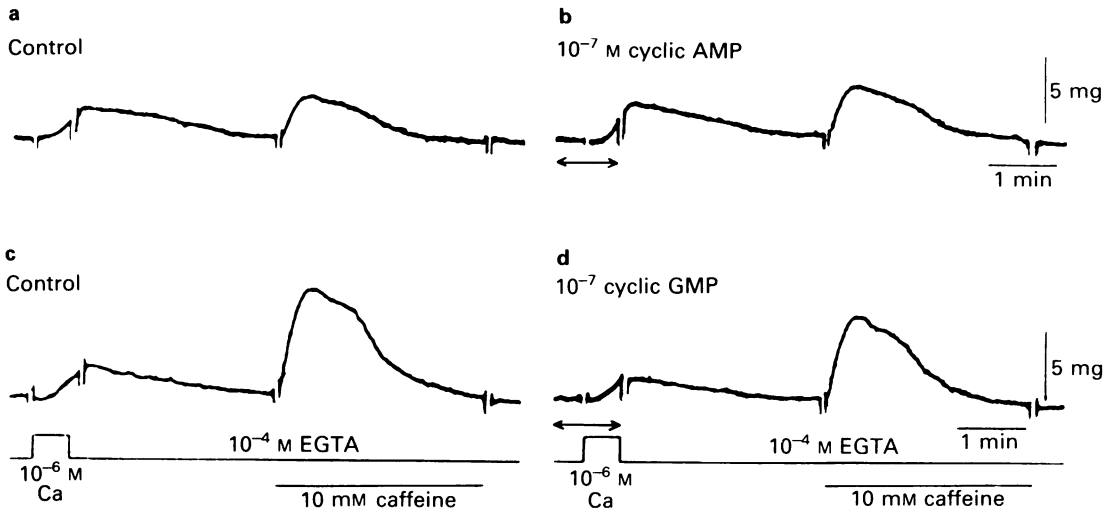

Figure 7 Effects of cyclic AMP or cyclic GMP on caffeine-induced contractions in skinned muscle tissues of the rabbit mesenteric artery. Before and during the administraon of 1 μM Ca for 30 s, either 0.1 μM cyclic AMP or cyclic GMP was given for 1 min. After a 3 min rinse with relaxing solution (containing 0.1 mM EGTA), 10 mM caffeine was added. (a) Control; (b) administration of cyclic AMP (0.1 μM). (c) Control; (d) administration of cyclic GMP (0.1 μM). Administration of cyclic nucleotides are indicated by arrows.

periods of administration or higher concentrations of Ca, cyclic AMP reduces the amount of stored Ca. On the other hand, cyclic GMP consistently reduces the amount of Ca stored in the cell.

Discussion

Roles of cyclic AMP and cyclic AMP-dependent protein kinase in relation to the action of isoprenaline

Since the noradrenaline-induced response in Ca-free solution modified the contraction subsequently evoked by caffeine, the sources of Ca producing the contractions to noradrenaline and caffeine are probably the same. However, caffeine appeared to release Ca more rapidly than noradrenaline as estimated from the shapes of the responses. It has already been shown that caffeine accelerates Ca extrusion from the cell more than noradrenaline. Neither noradrenaline nor caffeine itself modified the contraction evoked by Ca in skinned muscles, whereas caffeine did, but noradrenaline did not, release Ca from the storage site in skinned muscles (Itoh *et al.* 1982; 1983a,b). Noradrenaline activates the adrenoceptors distributed on the surface membrane and may produce a substrate through the phosphatidylinositol cascade, thus causing the release of Ca from storage sites (Mitchell, 1975).

In vascular smooth muscles, cyclic AMP contributes to the muscle relaxation through two different mechanisms; one is through stimulation of the Ca pump at the sarcolemmal membrane (Ca extrusion) and sarcoplasmic reticulum (Ca accumulation), and the other is through inhibition of phosphorylation of myosin light chain kinase. However, no consistent results have been obtained concerning the Ca transport mechanism at the sarcolemmal membrane, i.e. Ca uptake was increased by cyclic AMP and cyclic AMP-PK into the sarcolemma-enriched fraction extracted from smooth muscle cells (Webb & Bhalla, 1976; Nishikori *et al.* 1977; Thorens & Haeusler, 1978; Bhalla *et al.*, 1978; Brockbank & England, 1980). On the other hand, negative results on the action of these agents have also been reported (Allen, 1977; Sands & Mascali, 1978; Kreye & Schlicker, 1980). The inhibitory action of cyclic AMP and cyclic AMP-PK on myosin light chain kinase is, however, generally accepted (Adelstein & Eisenberg, 1980). Recently, Suematsu *et al.* (1984) demonstrated that Ca extrusion in the sarcolemma-rich fraction extracted from aortae was accelerated by the addition of cyclic GMP with cyclic GMP-PK or cyclic AMP with cyclic AMP-PK in the presence of ATP, while Ca accumulation into the sarcoplasmic reticulum-rich fraction was only accelerated by cyclic AMP with cyclic AMP-PK but not by cyclic GMP with cyclic GMP-PK.

In skinned muscles, administration of free Ca increased the amount of Ca stored in the cell. However, excess accumulation of Ca into the storage sites activates the Ca-induced Ca release mechanism, thereby reducing the amount of Ca stored in the cell (Itoh *et al.* 1982). In the present experiments, in order to observe the effects of cyclic AMP on the Ca store in skinned muscles, the amount of Ca stored in cells was estimated from the amplitude of the caffeine-induced contraction. Since cyclic AMP-PK inhibited the phosphorylation of myosin, cyclic AMP was applied in the absence of cyclic AMP-PK, i.e. 30 s incubation of Ca in the presence of cyclic AMP gave a greater increase in the amount of Ca, as estimated from the amplitude of the caffeine-induced contraction in skinned muscles, than in the absence of cyclic AMP, whereas 120 s incubation of Ca in the presence of cyclic AMP gave a greater reduction in the amount of Ca stored in cells than in the absence of cyclic AMP. Therefore, cyclic AMP, in relation to the action of isoprenaline, inhibited the phosphorylation of the myosin light chain kinase and accelerated both the Ca accumulation into the store site and also Ca transport through the sarcolemmal membrane.

In contractile proteins, after complex formation of Ca with calmodulin, the effects of cyclic AMP and cyclic AMP-PK on the phosphorylation of myosin are less pronounced (Conti & Adelstein, 1981). On the other hand, in intact muscle tissues, isoprenaline is more effective against the tonic component than the phasic component of the K-induced contraction (i.e. isoprenaline is more effective after Ca has bound with calmodulin). Thus the main action of isoprenaline is postulated to be due to uptake of Ca into the store site and partly due to Ca extrusion from cells, in addition to inhibition of phosphorylaton of myosin.

Roles of cyclic GMP and cyclic GMP-dependent protein kinase in relation to the action of nitroglycerine

Cyclic GMP accelerates Ca transport at the sarcolemmal membrane in various smooth muscles (aorta: Ives *et al.*, 1980; Rapoport *et al.*, 1982; Suematsu *et al.*, 1984; trachea: Hogaboom *et al.*, 1982). In the present experiments, with the addition of cyclic GMP plus cyclic GMP-PK, the pCa-tension relationship was inhibited at concentrations of pCa below 6, while cyclic AMP-PK, with cyclic AMP consistently inhibited the pCa-tension relationship at any given concentration of Ca below pCa 5.

In order to observe the effects of cyclic GMP on the Ca storage site, cyclic GMP was applied in the absence of cyclic GMP-PK to skinned muscle tissues, thus eliminating its inhibitory actions on the phosphorylation of myosin. Cyclic GMP consistently reduced the amount of Ca stored in skinned muscles, relative to the control amounts, after the administration of any given concentration of Ca.

When the effects of cyclic GMP were considered in relation to the actions of nitroglycerine, cyclic GMP with cyclic GMP-PK inhibited the contraction induced by low concentrations of Ca (below 1 μM) and reduced the amount of Ca stored in the cell. In intact muscle cells (isolated single cell pellets in Ca-free solution), nitroglycerine significantly accelerated the Ca extrusion in the presence of vasoconstrictor agents (Itoh *et al.*, 1983b). When nitroglycerine or 8-Br cyclic GMP was applied together with Ca to intact muscle tissues the resulting contractions induced by caffeine in Ca-free solution, by both conditioning and test stimuli, were consistently reduced (Figure 4A). These responses differed from those evoked by isoprenaline or db cyclic AMP, i.e. the amount of Ca stored in cells was reduced by nitroglycerine or 8-Br cyclic GMP. The main action of cyclic GMP seems to be acceleration of Ca extrusion at the sarcolemmal membrane. From the results obtained from intact muscle tissues and sarcolemma-rich fractions, it may be deduced that the acceleration of Ca extrusion by cyclic GMP plus cyclic GMP-PK or nitroglycerine reduces the amounts of Ca stored and free Ca in the myoplasm.

The minimum requirement of cyclic AMP or cyclic GMP for the regulation of Ca stored in cells under physiological conditions is not yet clarified. However, if cyclic nucleotides play an important role in the relaxant actions of nitroglycerine or isoprenaline, the amount of stored Ca as regulated by the cyclic nucleotides may indicate the major difference in actions of both agents.

Not only does nitroglycerine, but also high concentrations of stimulants (acetylcholine, histamine and others), increase the mount of cyclic GMP (Bar, 1984; Hardman, 1981). However, nitroglycerine relaxes the tissue whereas the above agonists produce contractions. If the main action of nitroglycerine is the production of cyclic GMP, and cyclic GMP primarily accelerates Ca extrusion, then nitroglycerine would reduce the free Ca and Ca stored in the cell, thus relaxing the tissue. On the other hand, while acetylcholine and the other agonists increase the amount of free Ca, simultaneously increased concentrations of cyclic GMP may activate Ca extrusion, thus preventing the damage to cells caused by excess concentrations of Ca.

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