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Mechanisms controlling caffeine-induced relaxation of coronary artery of the pig

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1 We studied the effects of caffeine on coronary artery smooth muscle of the pig by measuring changes in isometric tension, cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and transmembrane potential.

2 In the absence of tone, caffeine induced a concentration-dependent transient contraction of coronary artery strips, followed by sustained relaxation. Simultaneously with the relaxation, caffeine, 25 mM, hyperpolarized the smooth muscle cells by 7.7 ± 0.9 mV.

3 Caffeine caused a concentration-dependent relaxation of strips precontracted with 10^{-5} M acetylcholine (ACh). A supramaximal relaxing concentration of 25 mM caffeine produced an additional transient increase in $[Ca^{2+}]_i$ on the Ca^{2+} plateau of ACh tonic contraction, which was followed by a decrease in $[Ca^{2+}]_i$ to a level slightly below the basal concentration. This relaxation was accompanied by a hyperpolarization of 7.3 ± 0.9 mV.

4 KCl 120 mM (high K^+) contracted the strips with a concomitant depolarization of 38.6 ± 1.6 mV and sustained increase in $[Ca^{2+}]_i$. Caffeine caused a concentration-dependent relaxation of high K^+-induced contraction. Caffeine, 25 mM, decreased the Ca^{2+} plateau to a level that remained above the basal concentration of Ca^{2+} but did not change the membrane potential.

5 When strips were placed in a Ca^{2+}-free medium with EGTA 2 mM and, in addition, ACh was applied successively three times, both intracellular and extracellular mobilizable Ca^{2+} pools were depleted. In these conditions, phorbol 12,13 dibutyrate (PDBu) 10^{-7} M and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M contracted the strips. Caffeine (25 mM) inhibited these contractions with no change in $[Ca^{2+}]_i$.

6 Forskolin, 3×10^{-7} M, inhibited ACh induced-contraction but did not affect those induced by PDBu.

7 In conclusion, these results show that caffeine has multiple cellular effects. During caffeine-induced relaxation, $[Ca^{2+}]_i$, adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content and membrane potential are modified. The findings suggest, however, that these effects are secondary, and that caffeine acts mainly by another unknown mechanism, possibly involving a direct inhibition of the contractile apparatus.

Keywords: Caffeine, Ca^{2+}, smooth muscle, Ca^{2+} independent, coronary artery

Introduction

Caffeine has been widely used as a pharmacological tool to study contraction-relaxation cycles in smooth muscle cells. Caffeine causes transient contractions in smooth muscles by releasing Ca^{2+} from intracellular or extracellular stores (Ito & Kuriyama 1971; Sunano & Miyazaki, 1973; Casteels *et al.*, 1977; Itoh *et al.*, 1982; Karaki *et al.*, 1987; Matsumoto *et al.*, 1990). In addition to these contractile effects, caffeine has potent relaxing activities on precontracted smooth muscles (Leijten & van Breemen, 1984; Sato *et al.*, 1988; Ahn *et al.*, 1988). The mechanisms of these relaxations are not completely understood as caffeine has multiple effects on intracellular machinery: inhibition of Ca^{2+} influx (Leijten & van Breemen, 1984; Martin *et al.*, 1989), increase in Ca^{2+} extrusion (Ahn *et al.*, 1988), increase in cytosolic adenosine 3′,5′-cyclic monophosphate (cyclic AMP) by the inhibition of phosphodiesterase (Polson *et al.*, 1978; Fredholm *et al.*, 1979; Bray *et al.*, 1989). Cyclic AMP increases Ca^{2+} uptake in sarcoplasmic reticulum (Saida & van Breemen, 1984), inhibits myosin light chain kinase activity (Conti & Adelstein, 1980) and decreases Ca^{2+} influx (Abe & Karaki, 1988). All these mechanisms implicate Ca^{2+}. Indeed, a large number of contractile agonists mobilize extracellular or intracellular Ca^{2+}, but contractions independent of Ca^{2+} have been described, notably via protein kinase C activation with phorbol esters (Chatterjee & Tejada, 1986; Singer & Baker, 1987), prostaglandins (Bradley & Morgan, 1987; Heaslip & Sickels, 1989) or with okadaic acid (Ozaki *et al.*, 1987; Hirano *et al.*, 1989).

Our goal was to evaluate more fully the mechanisms by which caffeine induces vascular relaxation. During caffeine-induced relaxation, isometric tension, changes in $[Ca^{2+}]_i$ and smooth muscle cell membrane potential were measured. Caffeine was tested under conditions in which contractions were accompanied by an increase in $[Ca^{2+}]_i$ without changes in membrane potential (acetylcholine (ACh)-induced contraction), on contractions accompanied by an increase in $[Ca^{2+}]_i$ and depolarization (high K^+-induced contraction), and on contractions independent of Ca^{2+} (phorbol ester-induced contraction).

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Methods

Pharmacological experiments

The anterior left descending branch of pig coronary artery was taken from freshly killed pigs at the local slaughterhouse. The lumen was quickly rinsed with ice cold oxygenated Krebs solution of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8, glucose 10.1 and gassed with 95% O_2 : 5% CO_2 at pH 7.4. The 120 mM KCl (high K^+) solution was identical except for an equimolar substitution of KCl for NaCl. The artery was cut longitudinally and the endothelium was removed by gently rubbing with a cotton tip. Segments were cleaned of all adherent fat and connective tissue. Tension was measured in a 85 μl tissue bath (Mastrangelo & Mathison, 1983) by use of two silk threads tied to the extremities of the strip. One extremity was attached to the bottom of the bath and the other to a Grass force displacement transducer (FTO3C). Changes in isometric tension were amplified (Lectromed 3559) and recorded on a chart paper with polygraphs (W + W Electronics). Strips were continuously superfused with oxygenated Krebs solution (1.25 ml min^{-1}) maintained at 37°C. The muscles were stretched up to 10 mNewton (mN) and allowed

to stabilize for about 1 h. This tension was readjusted to 10 mN during stabilization and was taken as the baseline.

Cytosolic free Ca^{2+} measurements

Segments of about 5 cm long were cut longitudinally and stretched over a tissue chamber (20 μl). The endothelium and the adventitia were carefully removed at the place of observation which corresponded to the surface of the chambers. Here the strip was 215 ± 16 μm thick ($n = 15$). The tissue was loaded with Fura-2 AM (10 μM), the acetoxymethyl ester form of Fura-2 (Grynkiewicz *et al.*, 1985), for 2 h in a HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid)-buffered solution containing (mM): NaCl 145, KCl 5, $CaCl_2$ 1, $MgSO_4$ 0.5, NaH_2PO_4 1, HEPES 20 and glucose 10.1 at pH 7.4; 37°C. The tissue was then rinsed for 1 h in the solution used for pharmacological experiments (described above) but with a modified gas mixture of 75% N_2; 20% O_2 and 5% CO_2, since high O_2 concentrations greatly reduce the intensity of Fura-2 fluorescence (Becker & Fay, 1987). The artery was then fixed in the chamber to reduce movement and continuously perfused (1.5 ml min^{-1}). All changes in perfusate were achieved without the introduction of bubbles which would have produced artefacts. Furthermore, continuous perfusion ensured that drug concentrations remained stable and removed the dye from the extracellular fluid. The fluorescence emitted by the cells corresponded to the field of the lens (magnification 10×) and was observed through a quartz lamella with a Nikon inverted microscope (Diaphot) equipped with a P1 Photometer. We used dual excitation wavelengths of 340 and 380 nm which were changed by sliding manually the double filter holder about every minute and more rapidly when the changes in $[Ca^{2+}]_i$ occurred. As in pig coronary artery changes in $[Ca^{2+}]_i$ during contraction-relaxation cycles are slow, this method permitted us to evaluate indirectly absolute changes in $[Ca^{2+}]_i$. A neutral density reduction filter was placed in front of the 380 nm excitatory filter to obtain roughly the same fluorescence intensity as in the 340 nm trace. A continuous control recording was made separately for each of the two excitatory wavelengths. Changes in fluorescence were recorded on a W + W Electronics recorder and the $[Ca^{2+}]_i$ levels were estimated by calculating the ratio of the emission at 510 nm following excitation at 340 and 380 nm (Grynkiewicz *et al.*, 1985). The ratio was calculated each time the filters were alternated. The advantage of the dual excitation ratio method is that it corrects for instrument artefacts and reduces problems associated with photobleaching (Hall *et al.*, 1988).

Electrophysiological experiments

Mechanical tension and transmembrane potential were measured simultaneously. The endothelium was removed as described previously. Strips were incubated in a perspex tissue bath, continuously perfused with oxygenated Krebs solution (1.25 ml min^{-1}) maintained at 37°C. One end of each strip was pinned onto a silicon rubber (Silgard) surface, intimal side facing up. The other end was attached horizontally to the force transducer. The strip was stretched to a tension of 10 mN and allowed to stabilize for 30 min, during which time tension was readjusted to 10 mN. The membrane potential was measured with conventional glass microelectrodes (80 MΩ) filled with 3 M KCl. Cells near the pins were impaled to reduce movement artefacts.

Drugs

Fura-2 acetoxymethyl ester, acetylcholine, prostaglandin $F_{2\alpha}$, phorbol 12,13 dibutyrate (PDBu) and forskolin were from Sigma. Fura-2 AM was dissolved in dimethyl sulphoxide (DMSO) and stored frozen at −20°C. Final concentration of DMSO never exceeded 1%. Prostaglandins and PDBu were dissolved in ethanol.

Data and analysis

Results are expressed as means ± s.e.mean. Comparisons of the results were made by Student's unpaired t test; $P < 0.05$ was accepted as significantly different.

Results

Effects of caffeine on basal tension

In the absence of tone, caffeine induced a concentration-dependent transient contraction of coronary artery strips that was associated with a transient increase in $[Ca^{2+}]_i$ (Figure 1). The contraction was followed by relaxation which was maximal at a concentration of 10^{-3} M. Simultaneously with the relaxation, 25 mM caffeine hyperpolarized the cells: the membrane potential was −43.7 ± 2.3 mV ($n = 3$) before, and −51.3 ± 1.5 mV ($n = 3$) after treatment with caffeine (Figure 1).

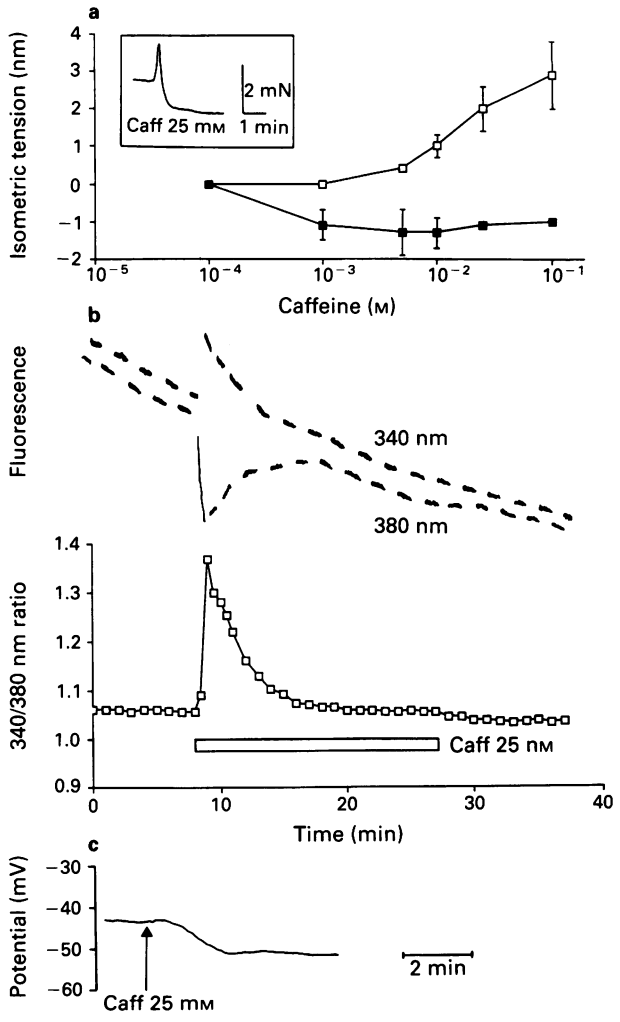

Figure 1 (a) Concentration-response curve of caffeine on the isometric tension of resting pig coronary artery strips. The zero value was related to the basal tension. Positive values represent contractions (□) and negative values represent relaxation (■) in relation to the basal tension. Points represent the mean and vertical bars the s.e.mean of 8 observations. The inset shows an original recording of the effect of 25 mM caffeine (Caff 25 mM). (b) Fluorescence signals of Fura-2 loaded strips were recorded at two excitation wavelengths, 340 and 380 nm. The breaks of the traces corresponded to the shifts of the excitation filters when they were changed from 340 to 380 nm. The ratio (340/380 nm) was calculated at each break and corresponded to an estimation of the changes in $[Ca^{2+}]_i$. (c) Recording of the smooth muscle cell transmembrane potential with conventional glass microelectrodes. These records are representative of 3 to 8 experiments.

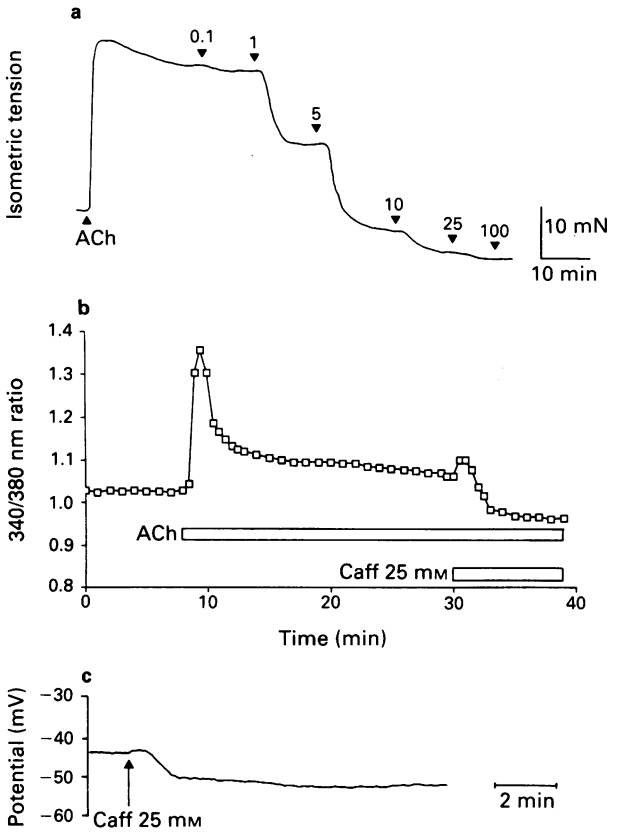

Figure 2 Effects of caffeine on strips precontracted with acetylcholine (ACh) 10^{-5} M. (a) A concentration-response curve with concentrations expressed in mM. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). (c) A recording of the smooth muscle cell transmembrane potential. These records are representative of 4 to 8 experiments.

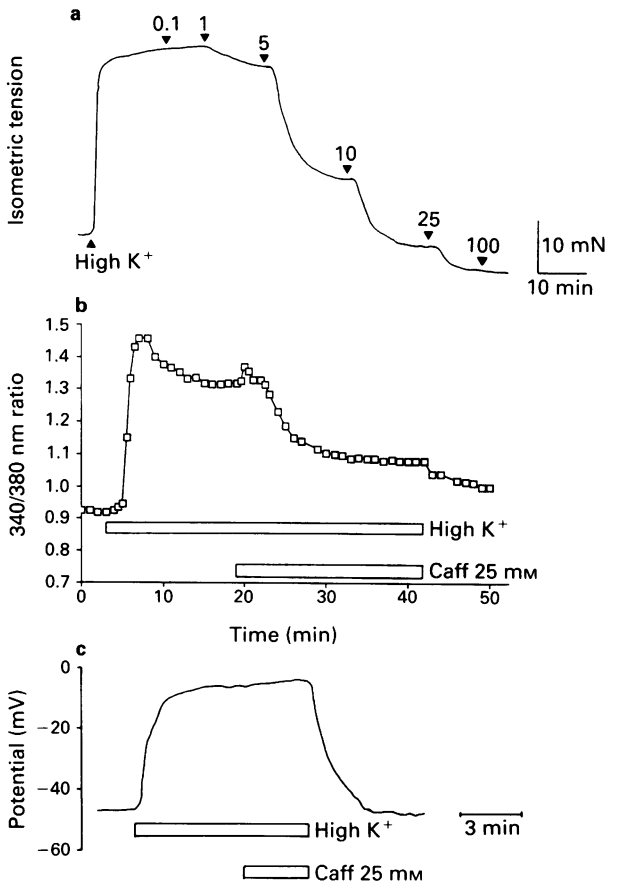

Figure 3 Effects of caffeine on strips precontracted with 120 mM KCl (High K^+). (a) A concentration-response curve with concentrations expressed in mM. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). (c) A recording of the smooth muscle cell transmembrane potential. These records are representative of 7 to 8 experiments.

Effects of caffeine on acetylcholine-induced contractions

ACh (10^{-5} M) induced a phasic contraction followed by a sustained tonic contraction (Figure 2). The $[Ca^{2+}]_i$ was maintained slightly above the basal concentration during the tonic contraction. ACh contracted the strip without changing the membrane potential (data not shown). When applied during the ACh-induced contraction, caffeine caused a concentration-dependent relaxation. A supramaximal relaxing concentration of caffeine (25 mM) produced an additional transient increase in $[Ca^{2+}]_i$ on the Ca^{2+} plateau and this was followed by a decrease in $[Ca^{2+}]_i$ to a level slightly below the basal concentration. This was accompanied by hyperpolarization of the cells from -45.8 ± 1.7 mV ($n = 4$) before to -53.0 ± 1.1 mV ($n = 4$) after treatment with caffeine (Figure 2). Moreover, when 25 mM caffeine was applied simultaneously with 10^{-5} M ACh, only a phasic contraction was obtained. However, preincubation with 25 mM caffeine for 30 min inhibited both phasic and sustained contractions induced by ACh (data not shown).

Effects of caffeine on high K^+-induced contractions

High K^+-induced contraction was associated with a sustained increase in $[Ca^{2+}]_i$ and a depolarization of 38.6 ± 1.4 mV ($n = 7$) (Figure 3). Caffeine induced a concentration-dependent relaxation of high K^+-induced contraction. At a supramaximal concentration, caffeine (25 mM) induced an additional transient increase in $[Ca^{2+}]_i$ which then fell and stabilized above the basal concentration but did not alter the membrane potential (Figure 3). When caffeine was applied simultaneously with high K^+, the depolarization obtained was similar to that obtained in the absence of caffeine (data not shown).

However, when 25 mM caffeine was applied simultaneously with high K^+, only a transient contraction was observed. Moreover, preincubation with 25 mM caffeine for 30 min totally inhibited high K^+-induced contractions (data not shown).

Caffeine was less potent in relaxing strips contracted with high K^+ than with ACh (Figure 4) but complete relaxation was obtained in each case with 25 mM caffeine.

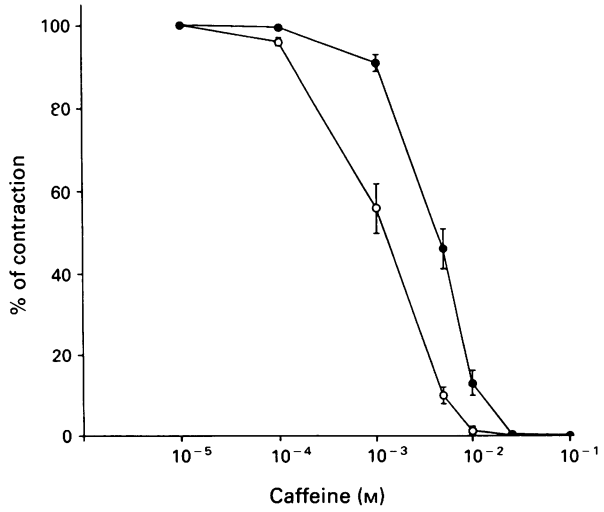

Figure 4 Concentration-response curve of the inhibitory effect of caffeine on strips precontracted with acetylcholine 10^{-5} M (○, $n = 8$) or KCl 120 mM (●, $n = 8$).

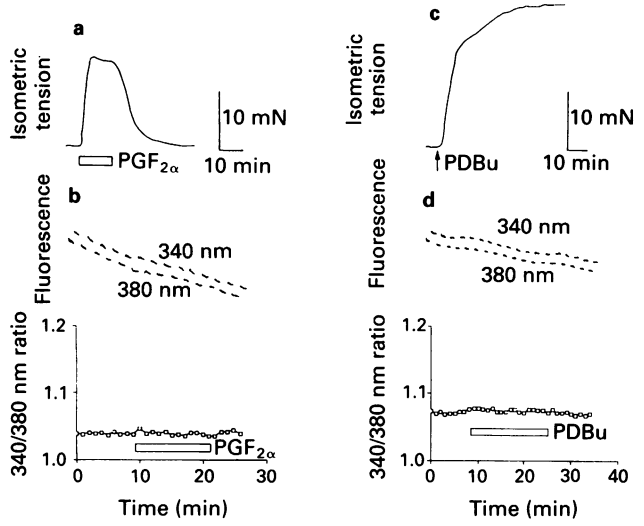

Figure 5 Effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M (a, b) and phorbol 12,13 dibutyrate 10^{-7} M (PDBu) (c, d) on isometric tension and $[Ca^{2+}]_i$ (ratio 340/380 nm).

Effects of caffeine on Ca^{2+}-independent contractions

PDBu (10^{-7} M) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M) contracted the strips without changing $[Ca^{2+}]_i$ (Figure 5) and 25 mM caffeine inhibited these contractions completely. When the strips were placed in a Ca^{2+}-free solution containing EGTA (2 mM) and ACh was applied successively three times,

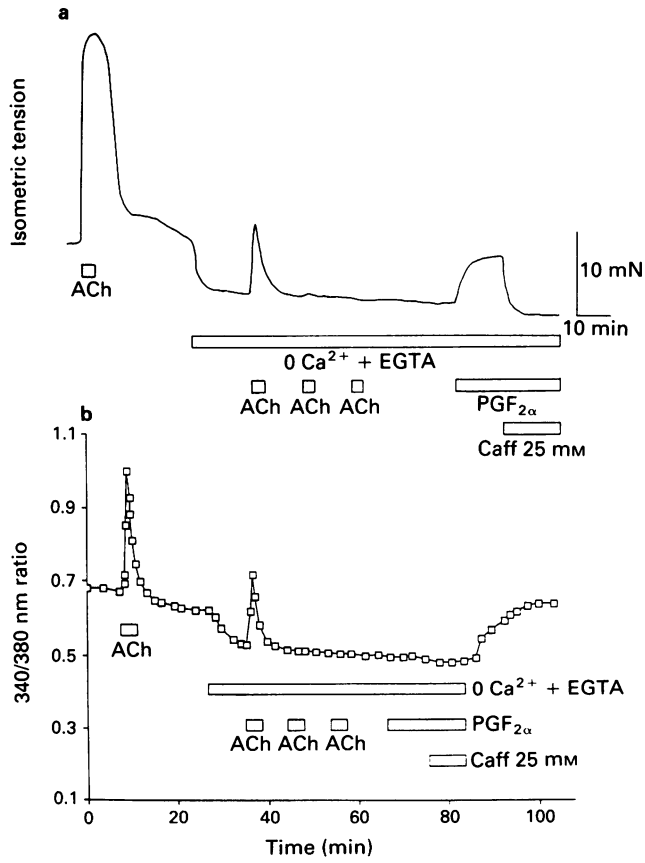

Figure 6 (a) Effects of caffeine (Caff) on isometric tension under conditions in which intracellular and extracellular mobilizable Ca^{2+} pools were depleted by applying acetylcholine (ACh) 10^{-5} M three times in succession in a Ca^{2+}-free EGTA (2 mM)-containing medium on strips precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). These records are representative of 4 to 8 experiments.

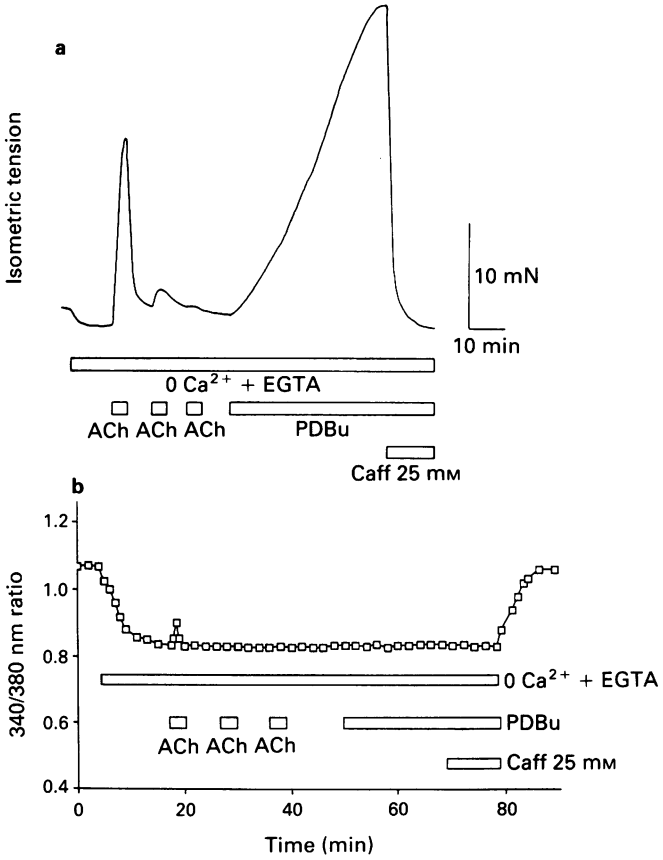

Figure 7 (a) Effects of caffeine (Caff) on isometric tension under conditions in which intracellular and extracellular mobilizable Ca^{2+} pools were depleted by applying acetylcholine (ACh) 10^{-5} M three times in succession in a Ca^{2+}-free EGTA (2 mM)-containing medium on strips precontracted with phorbol 12,13 dibutyrate (PDBu) 10^{-7} M. (b) Estimation of $[Ca^{2+}]_i$ changes (ratio 340/380 nm). These records are representative of 4 to 8 experiments.

both extracellular and intracellular mobilizable Ca^{2+} pools were depleted since ACh was no longer able to contract the strips and $[Ca^{2+}]_i$ was lower than the initial basal concentration (Figure 6). Under these conditions of Ca^{2+} depletion, $PGF_{2\alpha}$-induced contraction was reduced to $17 \pm 1\%$ ($n = 8$) of that obtained with the presence of extracellular Ca^{2+} (Figure 6). PDBu contracted the strips in the Ca^{2+}-free solution to the same degree of tension as that obtained in Ca^{2+}-containing solution, but contraction developed less rapidly (Figure 7). Under these conditions of Ca^{2+} depletion, caffeine (25 mM) inhibited $PGF_{2\alpha}$-induced contraction by $120 \pm 7\%$ ($n = 8$) and PDBu-induced contraction by $99 \pm 1\%$ ($n = 8$).

Effects of forskolin on acetylcholine and phorbol 12,13 dibutyrate-induced contractions

Forskolin 3×10^{-7} M, relaxed ACh (10^{-5} M)-induced contractions by $97 \pm 2\%$ ($n = 8$) in pig coronary artery but did not significantly affect PDBu-induced contractions, $P < 0.01$ ($n = 8$). Even at a concentration of 3×10^{-6} M, forskolin had no effect on PDBu-induced contractions (data not shown).

Discussion

Caffeine exhibited a rapid Ca^{2+}-dependent transient contraction followed by a sustained relaxation in pig coronary artery strips. ACh- and high K^+-induced contractions were inhibited by caffeine with no additional transient contraction and a lag before relaxation. This difference might be explained by the fact that in the latter case the contractions were already

maximal and caffeine was not able to induce a further contraction.

ACh-induced contraction was associated with a transient increase in $[Ca^{2+}]_i$ which was followed by a decrease to a plateau above the basal concentration but no change in membrane potential occurred. As caffeine hyperpolarized ACh-contracted strips and lowered $[Ca^{2+}]_i$, it is possible that hyperpolarization closed Ca^{2+} channels and led to a diminution of $[Ca^{2+}]_i$ resulting in relaxation. Indeed Martin *et al.* (1989) showed that caffeine inhibits Ca^{2+} influx by interacting with voltage-dependent Ca^{2+} channels. Hyperpolarization of pig coronary smooth muscle has already been observed in the relaxation induced by endothelium derived relaxing factors (Beny *et al.*, 1986; 1987; Beny & Brunet, 1988) and β-adrenoceptor agonists (Ito *et al.*, 1979). It is likely, however, that hyperpolarization is not essential since in the case of high K^+-induced contraction, where hyperpolarization was not possible, caffeine continued to produce relaxation. Nevertheless, in conditions in which hyperpolarization can occur, it is likely that closure of voltage-sensitive Ca^{2+} channels contributes to the relaxant action of caffeine.

Smooth muscle relaxation is believed to occur by a decrease in $[Ca^{2+}]_i$ and a dephosphorylation of myosin light chain (Gerthoffer & Murphy, 1983). Caffeine abolished the plateau phase of the increase in $[Ca^{2+}]_i$ induced by ACh, but only partially reversed that induced by high K^+. Surprisingly, under both conditions, complete relaxation was induced by caffeine. Moreover, caffeine completely relaxed contractions induced by $PGF_{2\alpha}$ or PDBu under conditions of extracellular and intracellular Ca^{2+} depletion where the $[Ca^{2+}]_i$ remained below the basal concentration. Consequently, decrease in $[Ca^{2+}]_i$ is not a necessary prerequisite for caffeine-induced relaxation of pig coronary smooth muscle. It is likely, however, that under conditions where caffeine does reduce $[Ca^{2+}]_i$, this contributes to the relaxant effect.

Caffeine has been shown to increase cyclic AMP content by inhibiting phosphodiesterase (Polson *et al.*, 1978; Fredholm *et al.*, 1979; Bray *et al.*, 1989). As cyclic AMP is known to cause relaxation of smooth muscle, we examined the effect of forskolin an activator of adenylate cyclase (Muller & Baer, 1983; Daly, 1984). A concentration of forskolin (3×10^{-7} M) that produces a concentration of cyclic AMP equivalent to that produced by 20 mM caffeine was used (Ozaki *et al.*, 1990). Caffeine completely relaxed strips precontracted with ACh or PDBu, but forskolin although able to relax ACh-induced contractions completely did not affect PDBu-induced contractions. These results contrast with those of Forder *et al.* (1985) who showed that 25 μM forskolin inhibits phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA)-induced contractions. The difference between that finding and ours may result from the use of different phorbol esters. Our data suggest that elevation of cyclic AMP content may not be a prerequisite for caffeine-induced relaxation. It is likely, however, that under conditions in which cyclic AMP elevation does occur, this contributes to the caffeine-induced relaxation.

In conclusion, caffeine has multiple cellular effects on pig coronary artery and is a powerful inhibitor of calcium-dependent and calcium-independent contractions. Caffeine-induced relaxation is accompanied by a decrease in $[Ca^{2+}]_i$, hyperpolarization and an increase in cyclic AMP (Polson *et al.*, 1978; Fredholm *et al.*, 1979; Bray *et al.*, 1989). These mechanisms are not observed under all conditions and the present observations suggest that they may be secondary. Our results and those of Ozaki *et al.* (1990) suggest that caffeine could mainly act through an unknown mechanism involving direct inhibition of actin-myosin filaments. Since contractions can be produced independently of Ca^{2+} and myosin light chain phosphorylation (Singer & Baker, 1987; Hoar & Kerrick, 1988), and since, under certain conditions, caffeine is able to induce relaxation without affecting $[Ca^{2+}]_i$, it is clear that much remains to be learned of the mechanisms that control contraction and relaxation in smooth muscle.

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