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Mechanism of CGRP-induced relaxation in rat intramural coronary arteries

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1 This study investigates the mechanism of CGRP-induced relaxation in intramural coronary arteries by determining the effect of CGRP on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) using FURA-2 technique.

2 CGRP concentration-dependently (10 pM – 100 nM) decreased the $[Ca^{2+}]_i$ and tension of coronary arteries precontracted with either U46619 or BAY K 8644, and also of resting coronary arteries in PSS. In 36 mM K^+-depolarized arteries, CGRP reduced only the tension without affecting the $[Ca^{2+}]_i$.

3 In 300 nM U46619- precontracted arteries, pretreatment with 10 μM thapsigargin significantly ($P<0.05$) attenuated the CGRP-induced reduction in the tension (but not $[Ca^{2+}]_i$).

4 In 300 nM U46619-precontracted arteries, pretreatment with either 100 nM charybdotoxin or 100 nM iberiotoxin or 10 nM felodipine significantly ($P<0.05$) attenuated the CGRP-induced reduction in both $[Ca^{2+}]_i$ and tension. In contrast, 1 μM glibenclamide did not affect the CGRP-induced responses in these coronary arteries.

5 In resting coronary arteries, only pretreatment with the combination of 1 μM glibenclamide and 100 nM charybdotoxin attenuated the CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension, suggesting a different mechanism of action for CGRP in resting coronary arteries.

6 We conclude that CGRP relaxes precontracted rat coronary arteries *via* three mechanisms: (1) a decrease in $[Ca^{2+}]_i$ by inhibiting the Ca^{2+} influx through membrane hyperpolarization mediated partly by activation of the large conductance Ca^{2+}-activated potassium channels, (2) a decrease in $[Ca^{2+}]_i$ presumably by sequestrating cytosolic Ca^{2+} into thapsigargin-sensitive Ca^{2+} storage sites and (3) a decrease in the Ca^{2+}-sensitivity of the contractile apparatus. In resting coronary arteries, however, there seems to be an interplay between different types of K^+ channels.
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Abbreviations: Ca^{2+}, calcium; Ca^{2+}- free PSS, substitution of $CaCl_2$ with 0.01 mM EGTA in physiological salt solution; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; K^+, potassium; K_{ATP}, ATP-sensitive potassium channel; K_{Ca}, large conductance Ca^{2+}-activated potassium channel; KPSS, equimolar substitution of NaCl with KCl in physiological salt solution; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PSS, physiological salt solution; Rat-αCGRP, rat-α calcitonin gene-related peptide; SR, sarcoplasmic reticulum

Introduction

Calcitonin gene-related peptide (CGRP) is a naturally occurring, 37 amino acid peptide that is generated by alternative splicing of the calcitonin gene transcripts (Amara *et al*., 1982). As a neurotransmitter CGRP is localized predominantly in sensory afferent nerves, which are primarily innervating the atrial muscle and the ventricular vasculature, indicating an important function of CGRP in the regulatory processes of coronary blood flow (Franco-Cereceda *et al*., 1987).

In vitro studies have shown that CGRP produces a positive inotropic effect in the atria of guinea-pig, rat and man as well as a potent vasodilatory effect in the coronary circulation of mammals including man (see Bell & McDermott, 1996 for review). CGRP is released from the perivascular sensory nerve endings in the wall of flow regulating intramural coronary arteries both *in vitro* (Franco-Cereceda & Lunberg, 1985; Franco-Cereceda *et al*., 1989, 1993) and *in vivo* (Kallner, 1998) during hypoxia and by low pH levels in the myocardium, thus suggesting a vasodilatory role under ischemic conditions.

A recent study on porcine coronary artery has shown that CGRP relaxes coronary arteries not only by inhibiting the influx of extracellular Ca^{2+} and the release of intracellular Ca^{2+}, but also by decreasing the Ca^{2+}-sensitivity of the contractile apparatus (Fukuizumi *et al*., 1996). In high K^+-depolarized coronary arteries, CGRP reduces the tensions without affecting the levels of intracellular calcium concentration ($[Ca^{2+}]_i$) (Kageyama *et al*., 1993; Fukuizumi *et al*., 1996). A number of studies have shown that CGRP reduces

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$[Ca^{2+}]_i$ and tension by increasing the cyclic AMP content in vascular smooth muscles (Kageyama *et al*., 1993; Ishikawa *et al*., 1993; Yoshimoto *et al*., 1998). The activation of potassium channels by CGRP has also been demonstrated by several studies (Nelson *et al*., 1990; Kitazono *et al*., 1993). However, two separate studies on isolated rat and porcine coronary arteries have not been able to demonstrate a possible involvement of ATP sensitive K^+ channels (K_{ATP}) in CGRP-induced vasodilatation (Prieto *et al*., 1991; Kageyama *et al*., 1993). It is well-known that CGRP can cause vasodilatation *via* a number of mechanisms. However, the mechanisms by which CGRP mediates the powerful vasodilatation in coronary resistance arteries are not fully known due to heterogeneity and complexity of the mechanism (Prieto *et al*., 1991). Thus, the purpose of the present study is to characterize the mechanism of CGRP-induced relaxation by means of FURA-2 technique in intramural coronary arteries from 3 months old male Sprague-Dawley rats.

Methods

Tissue preparation

All animal procedures were strictly within national laws and guidelines. Three months old male Sprague-Dawley rats were stunned by a blow on the head prior to exsanguination (decapitation). Then, the heart was rapidly removed and placed in ice-cold (4°C) oxygenated (95% O_2 and 5% CO_2) physiological salt solution (PSS) (composition see below) as previously described (Nyborg *et al*., 1987). Afterwards, arterial ring segments (1–2 mm long, one vessel per rat) were isolated from the same anatomical location in the distal, intramural part of the left coronary artery as previously described (Nyborg & Mikkelsen, 1985).

Measurement of force development

The coronary arteries were mounted as rings on two 40 μm stainless steel wires connected to a force transducer and a micrometer, respectively, in the organ bath of a small vessel myograph (Danish Myo Technology A/S, Aarhus, Denmark), which allowed direct determination of the isometric wall tension while the internal circumference of the vessels was controlled (Mulvany & Nyborg, 1980).

After mounting, the arteries were equilibrated in oxygenated PSS at 37°C, pH 7.4, for 30 min. The vessels were then stretched to their optimal lumen diameter $L_1 = 0.9 \times L_{100}$, where L_{100} is an estimate of the diameter the vessel would have under a passive transmural pressure of 100 mmHg (13.3 kPa (N m^{-2})), in order to secure maximal active force development (Nyborg *et al*., 1987). The effective vessel lumen diameter was calculated as L_1/π.

The vessels were repeatedly contracted, before and after loading with FURA-2, with KPSS (similar composition to PSS except that NaCl was exchanged with KCl on an equimolar basis) until reproducible contractions were recorded. The maximal contractile response of the vessels (ΔT_{max}) was then determined by measuring the difference in vessel wall tension (newton per meter of vessel wall, N m^{-1}) during contraction with activating solution (KPSS to which 10 μM serotonin and 10 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were added) and in Ca^{2+}-free PSS (Nyborg, 1991). Ca^{2+}-free PSS similar in composition to PSS except that $CaCl_2$ was replaced with 0.01 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). Cumulative CGRP concentration-response curves (10 pM – 100 nM) were constructed in one unit log molar increments.

FURA-2 loading procedure

The coronary arteries were loaded with the fluorescent $[Ca^{2+}]_i$ indicator dye (FURA-2) by incubating in oxygenated PSS containing 10 μM FURA-2/AM (an acetoxymethyl ester form of FURA-2), 0.2% (v v^{-1}) anhydrous dimethylsulphoxide (DMSO), 0.01% (v v^{-1}) pluronic F-127 and 0.03% (v v^{-1}) cremophor EL. Cremophore EL and pluronic F-127 (dispersing agents or non-ionic detergents) have been reported to improve the efficiency of loading with FURA-2 partly by promoting dye dispersion and partly by preventing FURA-2AM from precipitating (Roe *et al*., 1990).

The arteries were loaded twice for 30 min at 37°C. After loading, the arteries were washed with PSS and then equilibrated in PSS at 37°C for an additional 15 min before measurement of cytosolic Ca^{2+}. This provided sufficient time to wash away any extracellular FURA-2/AM and for intracellular esterases to cleave FURA-2AM into the active FURA-2. After loading with FURA-2, KPSS caused the same extent of tension development with the same time course as observed before loading (data not shown), thus indicating that loading the rat coronary arteries with FURA-2 does not affect the contractility, consistent with the previous findings in porcine coronary arteries (Abe *et al*., 1990; Hirano *et al*., 1990).

Measurement of $[Ca^{2+}]_i$

For Ca^{2+}-measurements, the myograph was placed on the stage of an inverted microscope (Leica LEITZ DMIRB, Germany) with optics for epifluorescence as previously described (Jensen *et al*., 1992). The coronary artery was illuminated with 340 and 380 nm light, and emitted light was passed through filters (500–530 nm) and detected by a photomultiplier. During the experiments, data (fluorescence signals and force signals) were captured and computer processed (FeliX™ program, Photon Technology International, Monmouth Junction, NJ, U.S.A.). All experiments with FURA-2 were performed in the dark. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the equation; $[Ca^{2+}]_i = K_d \beta \cdot [(R-R_{min})/(R_{max}-R)]$, with the assumption that the dissociation constant of FURA-2-Ca^{2+} complex, K_d, is 224 nM at 37°C (Grynkiewicz *et al*., 1985). The parameter is the ratio of emission at 380 nm excitation at maximum and minimum Ca^{2+} levels (corrected for background fluorescence signals). R_{min} and R_{max} were determined in each vessel at the end of the experiment by adding 40 μM ionomycin in calcium free buffer-solution (composition see below) and by using buffer-solution containing 5 mM Ca^{2+}(composition see below), respectively (Jensen *et al*., 1992). Before calculating the ratio (R) between emission at 340 nm illumination and emission at 380 nm illumination, background fluorescence signals were obtained by quenching the calcium-sensitive FURA-2 fluorescence with

20 mM Mn^{2+} at the end of each experiment (Roe *et al*., 1990; Jensen *et al*., 1992). The mean values ($n=28$) of R_{max}, R_{min}, and β were 4.76 ± 0.17, 1.19 ± 0.02 and 2.98 ± 0.14, respectively. The values in Ca^{2+}-free PSS (0.01 mM EGTA) and the plateau phases were designated to be 0 and 100% for both the $[Ca^{2+}]_i$ and force, respectively.

Drugs and solutions

PSS had the following composition (in mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4{\cdot}7H_2O$ 1.17, $CaCl_2{\cdot}2H_2O$ 1.5, ethylene diamine tetraacetic acid (EDTA) 0.027 and glucose 5.5 with pH adjusted to 7.4.

Solutions used for determination of R_{min} and R_{max} contained (in mM): 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) 5, KCl 125, $MgCl_2{\cdot}6H_2O$ 1.17 and glucose 5.5, and then either 2 mM EGTA or 5 mM $CaCl_2{\cdot}2H_2O$ was added, respectively.

Drugs used were U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) (Fluka Chemie AG, Switzerland), glibenclamide (Tocris Cookson, St. Louis, MO, U.S.A.), felodipine (Hässle AB, Sweden), charybdotoxin, iberiotoxin, BAY K 8644, ionomycin, pluronic F127, cremophor EL, rat-αCGRP, 5-hydroxytryptamine HCl (Sigma, St. Louis, MO, U.S.A.), FURA-2/AM (Molecular Probes, Leiden, The Netherlands) and prostaglandin $F_{2\alpha}$ (Dinoprost®, Upjohn, Belgium). FURA-2/AM was dissolved in loading mixture (anhydrous DMSO, pluronic F-127 and cremophor EL) just before loading. U46619, felodipine and BAY K 8644 were dissolved in ethanol at 10^{-2} M. Felodipine and BAY K 8644 were protected from light and experiments were performed in a darkened room. Charybdotoxin was dissolved in PSS containing 0.1% bovine serum albumin and iberiotoxin was dissolved in distilled water. Glibenclamide was dissolved in anhydrous DMSO at a concentration of 10^{-2} M. Rat-αCGRP was dissolved in distilled water at a concentration of 10^{-4} M. Stock solutions were stored at $-20^{\circ}C$ and dilutions were made just before experimentation.

Data analysis and statistics

Vessel responses are expressed either as a percentage of the precontraction tension or as a percentage of ΔT_{max} or as active vessel wall tension (N m^{-1}). The levels of $[Ca^{2+}]_i$ are given either as percentage of the plateau levels or as a percentage of the steady-state $[Ca^{2+}]_i$ level for ΔT_{max} or as absolute values (nM). Results are given as mean ± s.e.mean, ($n=$ number of vessels). Differences between mean values were analysed using either a two-tailed Student's *t*-test for paired or unpaired observations where appropriate or one-way analysis of variance (ANOVA). The level of significance was for all tests set to *P*-values less than 0.05.

Results

Effect of CGRP on the $[Ca^{2+}]_i$ and tension of coronary arteries during U46619- and 36 mM K^+- induced contraction

Figure 1 shows representative recordings of changes in the $[Ca^{2+}]_i$ and tension induced by rat-αCGRP (10 pM – 100 nM) in U46619-precontracted coronary arteries. Rat-αCGRP concentration-dependently reduced both the $[Ca^{2+}]_i$ and tension of the coronary arteries precontracted with 300 nM U46619. The application of U46619, a thromboxane A_2 analogue, induced a rapid increase in $[Ca^{2+}]_i$ and tension, which reached a plateau level within 5 min.

The mean steady-state level of the $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 was 204 ± 10 nM ($n=7$) and 2.42 ± 0.31 N m^{-1} ($n=7$), respectively. The maximal reduction in the $[Ca^{2+}]_i$ and tension induced by rat-αCGRP in U46619-precontracted arteries was $25\pm4\%$ or 43 ± 8 nM ($n=7$) and $63\pm8\%$ ($n=7$), respectively.

The mean steady-state level of the $[Ca^{2+}]_i$ in Ca^{2+}-free PSS and PSS was 28 ± 11 nM ($n=7$) and 103 ± 13 nM ($n=7$), respectively. When the bathing medium was changed from normal PSS (5.9 mM K^+) to KPSS (125 mM K^+), both the $[Ca^{2+}]_i$ and tension rapidly increased to reach (within 2 min) plateau levels of 331 ± 30 nM ($n=7$) and 2.40 ± 0.32 N m^{-1} ($n=7$), respectively.

In U46619-precontracted coronary arteries, the extent of CGRP-induced reduction in $[Ca^{2+}]_i$ was significantly ($r=0.86$; $P=0.0002$; $n=13$) correlated with the steady-state level of $[Ca^{2+}]_i$ induced by U46619 (Figure 2).

Figure 3 shows representative recordings of changes in the $[Ca^{2+}]_i$ and tension induced by rat-αCGRP (10 pM – 100 nM) in 36 mM K^+-depolarized coronary arteries. When the coronary arteries were depolarized with 36 mM K^+, the $[Ca^{2+}]_i$ and tension rapidly increased to reach (within 5–10 min) plateau levels of 203 ± 11 nM ($n=7$) and 1.14 ± 0.18 N m^{-1} ($n=7$), respectively. Rat-αCGRP concentration-dependently reduced the tension of the coronary arteries depolarized with 36 mM K^+ without causing a significant reduction in the $[Ca^{2+}]_i$. The maximal reduction in the $[Ca^{2+}]_i$ and tension induced by rat-αCGRP being $0.1\pm1.4\%$ or 3 ± 2 nM ($n=7$) and $40\pm2\%$ ($n=7$), respectively.

There was no significant difference in the plateau $[Ca^{2+}]_i$ levels between 36 mM K^+-depolarized coronary arteries and 300 nM U46619-precontracted coronary arteries. However,

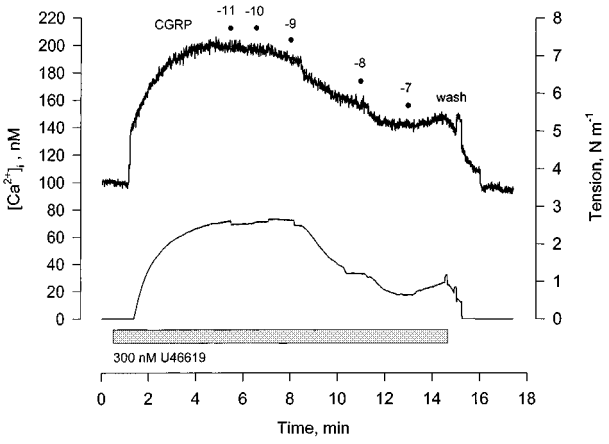

Figure 1 Representative recordings showing the effects of rat-αCGRP (10 pM – 100 nM) on the $[Ca^{2+}]_i$ (the curve above) and tension (the curve given below) of rat coronary arteries during the contraction with 300 nM U46619. The levels of $[Ca^{2+}]_i$ (nM) and tension (N m^{-1}) induced by U46619 are given as absolute values. The CGRP was applied cumulatively 5 min after the application of U 46619. Points represent application of CGRP.

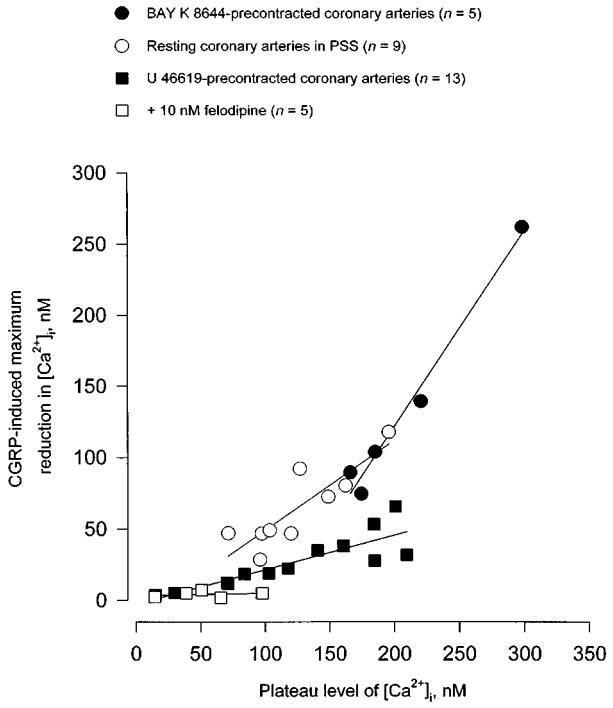

Figure 2 Relationship between the initial steady-state $[Ca^{2+}]_i$ level (nM) and the rat-αCGRP-induced maximal reduction in $[Ca^{2+}]_i$ (nM) under four different conditions: (1) BAY K 8644-precontracted coronary arteries, (2) resting coronary arteries in PSS, (3) U46619-precontracted coronary arteries (seven arteries precontracted in normal PSS and six arteries precontracted in Ca^{2+}-free PSS (0.01 mM EGTA) to which Ca^{2+} was added (0.1–0.5 mM) and (4) U46619-precontracted coronary arteries pretreated with 10 nM felodipine. The relationship was analysed by linear regression analysis.

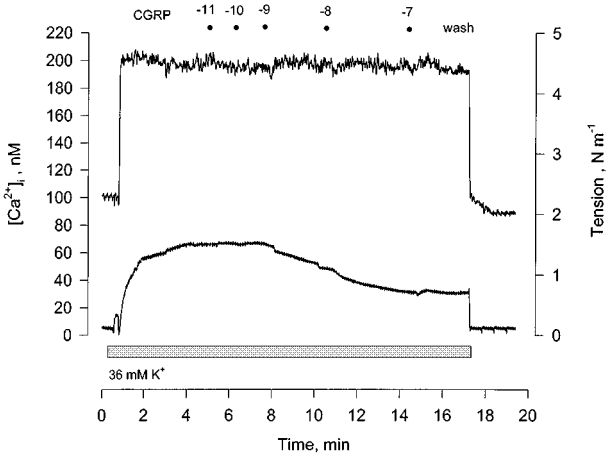

Figure 3 Representative recordings showing the effects of rat-αCGRP (10 pM–100 nM) on the $[Ca^{2+}]_i$ (the curve above) and tension (the curve given below) of rat coronary arteries during the depolarization with 36 mM K^+. The levels of $[Ca^{2+}]_i$ (nM) and tension (N m^{-1}) induced by 36 mM K^+ are given as absolute values. The CGRP was applied cumulatively 5 min after the application of 36 mM K^+. Points represent application of CGRP.

the steady-state contraction (N m^{-1}) induced by 300 nM U46619 was significantly ($P<0.01$) higher than that induced by 36 mM K^+ (Figure 4).

The extent of the CGRP-induced decrease in the tension was significantly ($P<0.05$) lower during 36 mM K^+-induced depolarization compared to those during precontraction with 300 nM U46619. Mean lumen diameter of coronary arteries was 208 ± 14 μm ($n=7$).

Effect of 10 μM thapsigargin on CGRP-induced reduction in the $[Ca^{2+}]_i$ and tension of U46619-precontracted coronary arteries

In these studies, we investigated whether or not the CGRP-induced reduction in the $[Ca^{2+}]_i$ was partly mediated by the uptake of cytosolic Ca^{2+} into the intracellular storage sites by using thapsigargin, a selective inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al*., 1990).

Thapsigargin significantly attenuated the CGRP-induced decrease in tension but not in $[Ca^{2+}]_i$ (Figure 5). Incubation of coronary arteries with 10 μM thapsigargin for 15 min caused a significant increase in the resting $[Ca^{2+}]_i$ of coronary arteries from 90 ± 11 nM to 134 ± 8 nM (Paired *t*-test, $P=0.009$; $n=6$). However, the tension of coronary arteries was not significantly affected by this treatment.

The maximal relaxation induced by rat-αCGRP before and after treatment with 10 μM thapsigargin was $64\pm9\%$ and $32\pm6\%$ (paired *t*-test, $P=0.0393$; $n=6$), respectively. The CGRP-induced decrease in $[Ca^{2+}]_i$ before and after treatment with 10 μM thapsigargin was $22\pm3\%$ or 42 ± 6 nM ($n=6$) and $20\pm3\%$ or 30 ± 6 nM ($n=6$), respectively.

The mean steady-state level of the $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 before and after treatment with 10 μM thapsigargin was 224 ± 15 nM and 2.52 ± 0.43 N m^{-1} ($n=6$) vs 194 ± 15 nM and 2.30 ± 0.32 N m^{-1} ($n=6$), respec-

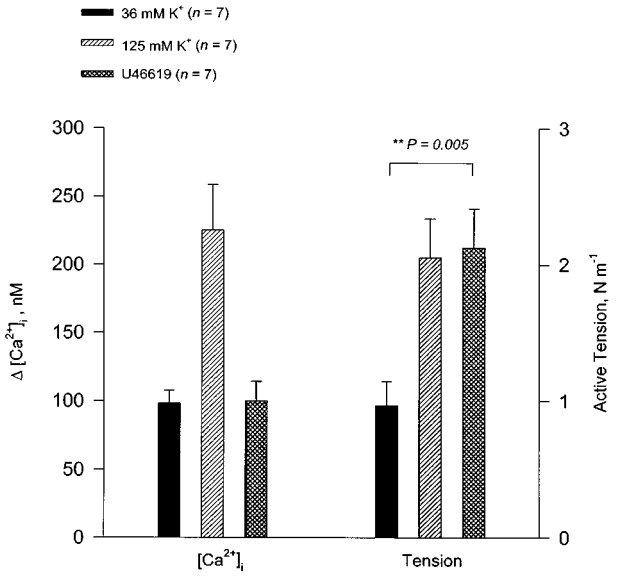

Figure 4 The increase in the $[Ca^{2+}]_i$ (nM) and tension (N m^{-1}) induced by 36 mM K^+, 125 mM K^+ (KPSS) and 300 nM U46619 in rat coronary arteries. The increase in $[Ca^{2+}]_i$ and tension are calculated as differences between the steady-state levels during the contraction and the resting levels in PSS. A paired two-tailed *t*-test was used to compare the mean values (**$P<0.01$; tension: 36 mM K^+ vs U46619). The bars represent mean values and the error bars indicate ± s.e.mean.

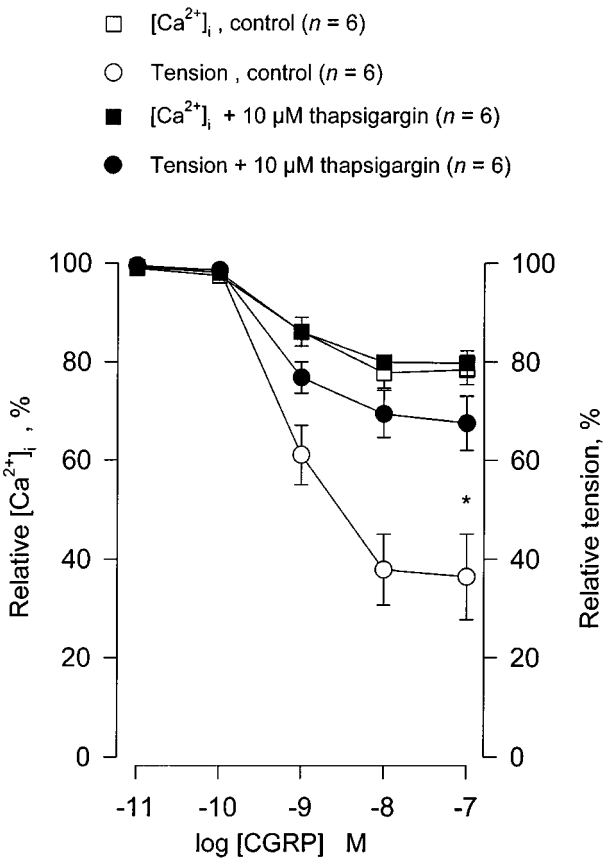

Figure 5 The effect of thapsigargin on the rat-αCGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of rat coronary arteries precontracted with U46619. Relative responses are given as percentages of the initial steady-state levels of $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 just before the vessels were challenged with rat-αCGRP. Points represent mean values and vertical bars indicate ± s.e.mean where this value exceeds the size of symbol. A paired two-tailed *t*-test was used to compare the mean values (*$P<0.05$).

tively. Mean lumen diameter of coronary arteries was 211 ± 10 μm ($n=6$).

Depletion of Ca^{2+}-stores by 10 μM thapsigargin

Two series of experiments were used in order to investigate whether the Ca^{2+}- stores were really depleted during the 15 min incubation with thapsigargin, In the first series, the coronary arteries were washed once with calcium-free PSS (0.01 mM EGTA) and then incubated in calcium-free PSS (0.01 mM EGTA) for 1 min. Afterwards, the coronary arteries were challenged with 10 μM serotonin (5-HT) and $[Ca^{2+}]_i$ spikes were measured. The second series of experiments were similar to the first series except that the coronary arteries were first preincubated in PSS with 10 μM thapsigargin for 15 min prior to incubation in the calcium-free PSS and subsequent activation by 10 μM 5-HT.

Again, incubation of coronary arteries with 10 μM thapsigargin for 15 min caused a significant increase in the resting $[Ca^{2+}]_i$ of coronary arteries kept in PSS from 96 ± 6 nM to 130 ± 6 nM (Paired *t*-test, $P=0.0009$; $n=4$). The tension of coronary arteries was not significantly affected by this treatment. The mean steady-state level of the $[Ca^{2+}]_i$ in Ca^{2+}-free PSS was 50 ± 9 nM ($n=4$).

Preincubation with 10 μM thapsigargin for 15 min caused a significant depletion of Ca^{2+}-stores. The $[Ca^{2+}]_i$ spikes induced by 10 μM 5-HT in calcium-free PSS (0.01 mM EGTA) were 185 ± 17 nM or 45 ± 2% and 48 ± 7 nM or 11 ± 2% of the steady-state $[Ca^{2+}]_i$ level for ΔT_{max} ($P=0.0003$; $n=4$), without and with thapsigargin preincubation, respectively. The maximal tension induced by 10 μM 5-HT in calcium-free PSS (0.01 mM EGTA) was 2.33 ± 0.06 N m^{-1} or 54 ± 5% of ΔT_{max} and 0.04 ± 0.02 N m^{-1} or 1.0 ± 0.7% of ΔT_{max} ($P<0.0001$; $n=4$), without and with thapsigargin preincubation, respectively.

Effect of 10 nM felodipine on CGRP-induced reduction in the $[Ca^{2+}]_i$ and tension of U46619-precontracted coronary arteries

Felodipine significantly ($P<0.01$) inhibited the CGRP-induced decrease in the $[Ca^{2+}]_i$ of U46619-precontracted coronary arteries (Figure 2). The CGRP-induced reduction in the $[Ca^{2+}]_i$ was 10 ± 3% or 3 ± 1 nM ($n=5$). Neither rat-αCGRP nor U46619 (0.65 ± 0.35 N m^{-1}, $P=0.1369$; $n=5$) significantly affected the tension of coronary arteries preincubated with 10 nM felodipine.

The $[Ca^{2+}]_i$ of coronary arteries kept in PSS gradually declined (within 2 min) from 94 ± 14 nM to reach a steady state level of 32 ± 10 nM ($n=5$). After incubation of coronary arteries with 10 nM felodipine for 15 min, the mean steady-state level of the $[Ca^{2+}]_i$ induced by 300 nM U46619 was 54 ± 14 nM ($n=5$). Mean lumen diameter of coronary arteries was 234 ± 13 μm ($n=5$).

In order to determine whether the slight reduction in $[Ca^{2+}]_i$ by rat-αCGRP was caused by felodipine or by lower plateau levels of the $[Ca^{2+}]_i$, the coronary arteries were incubated in calcium-free PSS (0.01 mM EGTA) for 2 min in the presence of 300 nM U46619 and then calcium (0.1–0.5 mM) was added extracellulary to reach the same plateau levels of the $[Ca^{2+}]_i$ as seen with felodipine.

Under this condition, CGRP concentration-dependently reduced the $[Ca^{2+}]_i$. Mean steady-state level of the $[Ca^{2+}]_i$ induced by 300 nM U46619 was 62 ± 13 nM ($n=6$), and CGRP-induced reduction in the $[Ca^{2+}]_i$ was 50 ± 4% or 13 ± 3 nM ($n=6$). Mean lumen diameter of coronary arteries was 245 ± 11 μm ($n=6$).

Effect of CGRP on the $[Ca^{2+}]_i$ and force of coronary arteries during contraction with 100 nM BAY K 8644, a selective activator of voltage-dependent calcium channels

The rat-αCGRP concentration-dependently decreased both the $[Ca^{2+}]_i$ and tension of coronary arteries precontracted with 100 nM BAY K 8644, a selective activator of voltage-dependent calcium channel (Franckowiak *et al.*, 1985) (Figure 6). The $[Ca^{2+}]_i$ and tension rapidly increased to reach (within 5 min) mean plateau levels of 209 ± 24 nM ($n=5$) and 2.51 ± 0.53 N m^{-1} ($n=5$), respectively.

The maximal reduction in $[Ca^{2+}]_i$ and tension induced by rat-αCGRP in BAY K 8644-precontracted coronary arteries was 56 ± 5% or 108 ± 13 nM ($n=5$) and 88 ± 2% ($n=5$), respectively. In BAY K 8644-precontracted coronary arteries,

the extent of CGRP-induced reduction in $[Ca^{2+}]_i$ was significantly ($r=0.99$; $P=0.0014$; $n=5$) correlated with the steady-state level of $[Ca^{2+}]_i$ induced by BAY K 8644 (Figure 2). Mean lumen diameter of coronary arteries was 216 ± 14 μm ($n=5$).

Effect of 1 μM glibenclamide on CGRP-induced reduction in the $[Ca^{2+}]_i$ and tension of U46619-precontracted coronary arteries

Pretreatment with glibenclamide, a selective inhibitor of ATP-sensitive potassium channels (K_{ATP} channels), did not have any significant effect on the CGRP-induced decrease in $[Ca^{2+}]_i$ or tension of U46619-precontracted coronary arteries (Figure 7). Incubation of coronary arteries with 1 μM glibenclamide for 15 min did not affect the $[Ca^{2+}]_i$ or tension of coronary arteries.

The maximal relaxation induced by rat-αCGRP before and after treatment with 1 μM glibenclamide was 55 ± 1% and 62 ± 7% ($n=4$), respectively. The CGRP-induced decrease in $[Ca^{2+}]_i$ before and after treatment with 1 μM glibenclamide was 31 ± 3% or 47 ± 5 nM ($n=4$) and 35 ± 2% or 45 ± 7 nM ($n=4$), respectively.

The mean steady-state level of the $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 before and after treatment with 1 μM glibenclamide was 191 ± 4 nM and 2.38 ± 0.31 N m^{-1} ($n=4$) vs 174 ± 2 nM and 1.97 ± 0.30 N m^{-1} ($n=4$), respectively. Mean lumen diameter of coronary arteries was 192 ± 12 μm ($n=4$).

Effect of 100 nM charybdotoxin or 100 nM iberiotoxin on CGRP-induced reduction in the $[Ca^{2+}]_i$ and tension of U46619-precontracted coronary arteries

Pretreatment with either charybdotoxin or iberiotoxin, selective inhibitors of large conductance calcium-dependent potassium channels (K_{Ca}), significantly ($P<0.05$) attenuated the rat-αCGRP-induced decrease in both $[Ca^{2+}]_i$ and tension of U46619-precontracted coronary arteries (Figure 8).

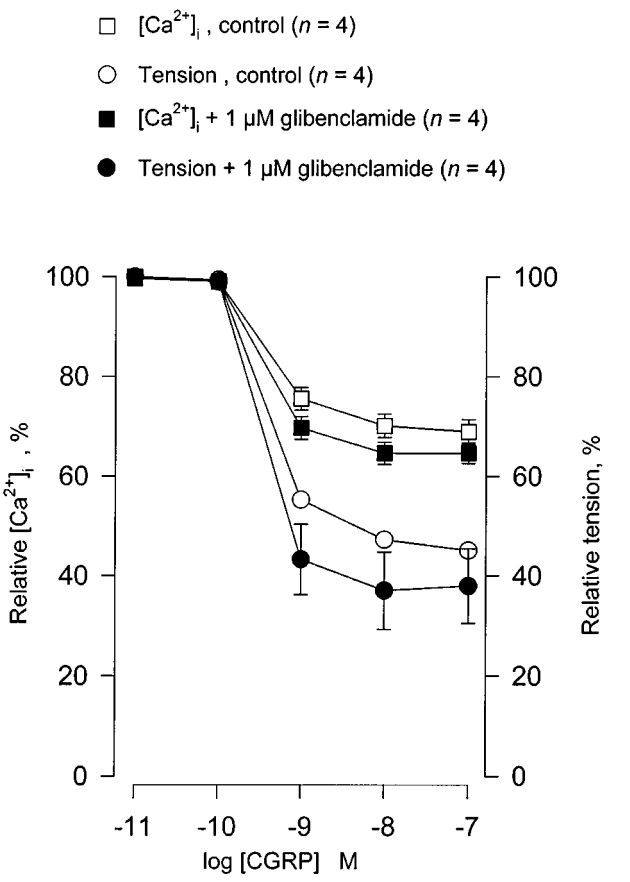

Figure 7 The effect of glibenclamide on the rat-αCGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of rat coronary arteries precontracted with U46619. Relative responses are given as percentages of the initial steady-state levels of $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 just before the vessels were challenged with rat-αCGRP. Points represent mean values and vertical bars indicate ± s.e.mean where this value exceeds the size of symbol.

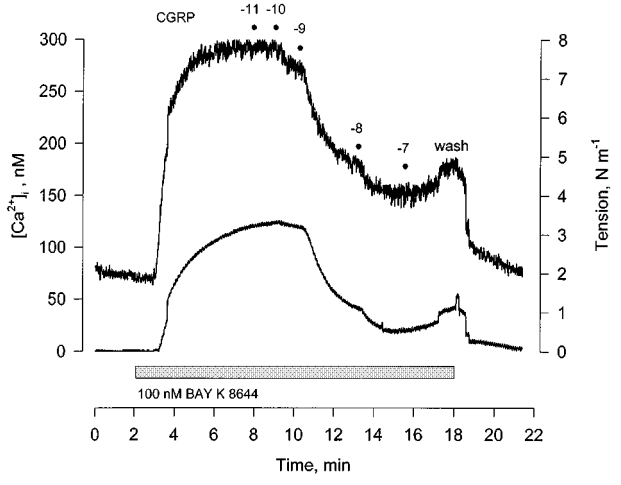

Figure 6 Representative recordings showing the effects of rat-αCGRP (10 pM – 100 nM) on the $[Ca^{2+}]_i$ (the curve above) and tension (the curve given below) of rat coronary arteries during the contraction with 100 nM BAY K 8644. The levels of $[Ca^{2+}]_i$ (nM) and tension (N m^{-1}) induced by BAY K 8644 are given as absolute values. The CGRP was applied cumulatively 6 min after the application of 100 nM BAY K 8644. Points represent application of CGRP.

Effect of charybdotoxin

Incubation of coronary arteries with 100 nM charybdotoxin for 15 min caused a significant increase in the resting $[Ca^{2+}]_i$ of coronary arteries from 127 ± 4 nM to 151 ± 6 nM (Paired *t*-test, $P=0.0123$; $n=4$). However, the tension of coronary arteries was not affected by this treatment.

The maximal relaxation induced by rat-αCGRP before and after treatment with 100 nM charybdotoxin was 70 ± 8% and 30 ± 8% ($P=0.0001$; $n=4$), respectively. The CGRP-induced decrease in $[Ca^{2+}]_i$ before and after treatment with 100 nM charybdotoxin was 40 ± 5% or 52 ± 3 nM ($n=4$) and 14 ± 3% or 15 ± 3 nM ($P=0.0051$; $n=4$), respectively (Figure 8a).

The mean steady-state level of the $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 before and after treatment with 100 nM charybdotoxin was 209 ± 4 nM and 2.69 ± 0.37 N m^{-1} ($n=4$) vs 188 ± 2 nM and 2.69 ± 0.39 N m^{-1} ($n=4$), respec-

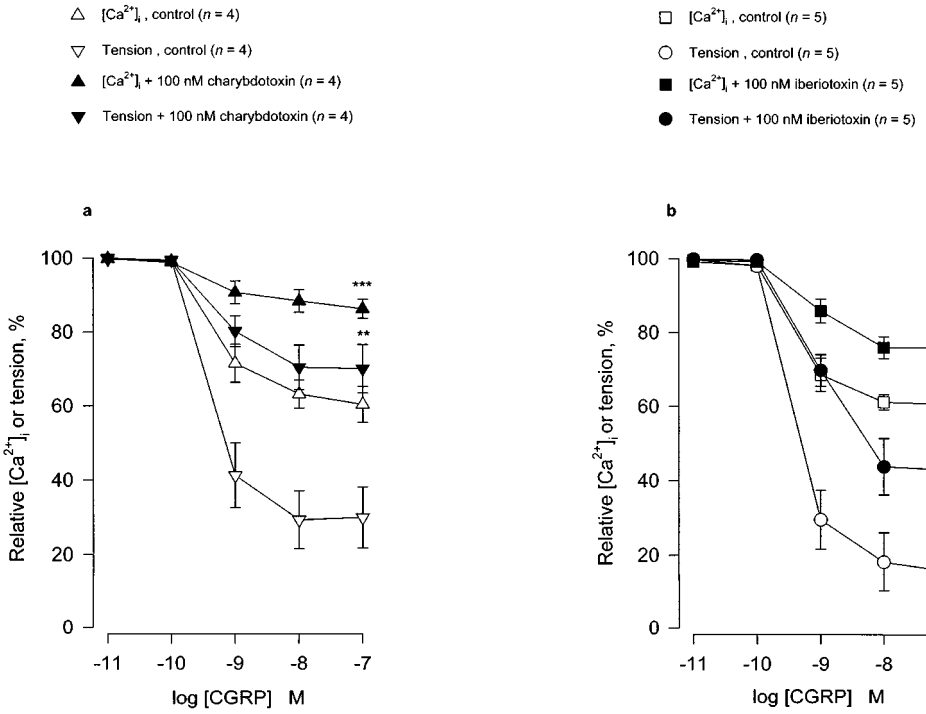

Figure 8 The effect of (a) 100 nM charybdotoxin and (b) 100 nM iberiotoxin on the rat-αCGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of rat coronary arteries precontracted with U46619. Relative responses are given as percentages of the initial steady-state levels of $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 just before the vessels were challenged with rat-αCGRP. Points represent mean values and vertical bars indicate ± s.e.mean where this value exceeds the size of symbol. A paired two-tailed *t*-test was used to compare the mean values ($*P<0.05$, $**P<0.01$, $***P<0.001$).

tively. Mean lumen diameter of coronary arteries was 210 ± 7 μm ($n=4$).

Effect of iberiotoxin

Incubation of coronary arteries with 100 nM iberiotoxin for 15 min caused a significant increase in the resting $[Ca^{2+}]_i$ of coronary arteries from 93 ± 6 nM to 129 ± 9 nM (paired *t*-test, $P=0.0048$; $n=5$). However, the tension of coronary arteries was not affected by this treatment.

The maximal relaxation induced by rat-αCGRP before and after treatment with 100 nM iberiotoxin was 86 ± 6% and 56 ± 8% ($P=0.0203$; $n=5$), respectively. The CGRP-induced decrease in $[Ca^{2+}]_i$ before and after treatment with 100 nM iberiotoxin was 39 ± 2% or 61 ± 3 nM ($n=5$) and 24 ± 3% or 41 ± 3 nM ($P=0.0043$; $n=5$), respectively (Figure 8b).

The mean steady-state level of the $[Ca^{2+}]_i$ and tension induced by 300 nM U46619 before and after treatment with 100 nM iberiotoxin was 197 ± 6 nM and 2.60 ± 0.28 N m^{-1} ($n=5$) vs 184 ± 6 nM and 2.50 ± 0.31 N m^{-1} ($n=5$), respectively. Mean lumen diameter of coronary arteries was 222 ± 6 μm ($n=5$).

Effect of CGRP on the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries in PSS

The rat-αCGRP concentration-dependently decreased both the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries kept in PSS. The maximal reduction in the resting $[Ca^{2+}]_i$ and basal tension induced by rat-αCGRP was 65 ± 4% or 65 ± 9 nM ($n=9$) and 81 ± 8% ($n=9$), respectively. The mean steady-state level of the $[Ca^{2+}]_i$ in calcium-free PSS, PSS and KPSS was 25 ± 5 nM, 125 ± 13 nM and 257 ± 18 nM ($n=9$), respectively.

Again, we found a significant ($r=0.86$; $P=0.003$; $n=9$) correlation between the steady-state resting level of $[Ca^{2+}]_i$ and the extent of CGRP-induced reduction in the resting $[Ca^{2+}]_i$ (Figure 2). Mean lumen diameter of coronary arteries was 232 ± 10 μm ($n=9$).

Effect of 100 nM charybdotoxin on CGRP-induced decrease in the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries in PSS

Pretreatment with 100 nM charybdotoxin did not have any significant effect on CGRP-induced reduction in the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries kept in PSS (Figure 9). The maximal relaxation induced by CGRP before and after treatment with 100 nM charybdotoxin in resting coronary arteries was 82 ± 4% and 81 ± 4% ($n=4$), respectively.

The CGRP-induced decrease in the resting $[Ca^{2+}]_i$ before and after treatment with 100 nM charybdotoxin was 65 ± 5% or 57 ± 8 nM ($n=4$) and 67 ± 6% or 63 ± 10 nM ($n=4$), respectively. The mean steady-state resting level of the $[Ca^{2+}]_i$ and tension in coronary arteries before and after treatment with 100 nM charybdotoxin was 108 ± 7 nM and 0.45 ± 0.23 N m^{-1} ($n=4$) vs 129 ± 10 nM and 0.46 ± 0.21 N m^{-1} ($n=4$), respectively. Mean lumen diameter of coronary arteries was 226 ± 22 μm ($n=4$).

Effect of 1 μM glibenclamide on CGRP-induced decrease in the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries in PSS

Pretreatment with 1 μM glibenclamide did not have any significant effect on CGRP-induced reduction in the resting $[Ca^{2+}]_i$ or basal tension of coronary arteries kept in PSS (Figure 9).

The maximal relaxation induced by CGRP before and after treatment with 1 μM glibenclamide in resting coronary arteries was 80±4% and 82±4% ($n=4$), respectively. The CGRP-induced decrease in the resting $[Ca^{2+}]_i$ before and after treatment with 1 μM glibenclamide was 58±9% or 45±8 nM ($n=4$) and 62±10% or 43±10 nM ($n=4$), respectively.

The mean steady-state resting level of the $[Ca^{2+}]_i$ and tension in coronary arteries before and after treatment with 1 μM glibenclamide was 106±11 nM and 0.47±0.10 N m^{-1} ($n=4$) vs 97±8 nM and 0.40±0.05 N m^{-1} ($n=4$), respectively. Mean lumen diameter of coronary arteries was 200±10 μm ($n=4$).

Effect of CGRP on the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries in PSS in the presence of both 1 μM glibenclamide and 100 nM charybdotoxin

Pretreatment at the same time with both 1 μM glibenclamide and 100 nM charybdotoxin significantly ($P<0.01$) attenuated the CGRP-induced reduction in the resting $[Ca^{2+}]_i$ and basal tension of coronary arteries kept in PSS (Figure 9).

The maximal relaxation induced by CGRP in resting coronary arteries before and after treatment with the two potassium-channel blockers was 82±5% and 60±4% (paired t-test, $P=0.0096$; $n=5$), respectively.

The CGRP-induced decrease in the resting $[Ca^{2+}]_i$ before and after treatment with the two potassium-channel blockers was 66±7% or 46±9 nM and 23±3% or 29±6 nM (paired t-test, $P=0.0026$; $n=5$), respectively. The mean steady-state level of the $[Ca^{2+}]_i$ and tension in resting coronary arteries before and after treatment with the two potassium-channel blockers was 99±9 nM and 0.42±0.19 N m^{-1} ($n=5$) vs 140±13 nM and 1.17±0.26 N m^{-1} ($n=5$), respectively. Mean lumen diameter of coronary arteries was 224±10 μm ($n=5$).

Discussion

The effects of CGRP on the levels of $[Ca^{2+}]_i$ and tension in rat coronary artery

Our study shows that CGRP concentration-dependently reduced both the $[Ca^{2+}]_i$ and tension not only during contraction with U46619 or BAY K 8644, but also in resting coronary arteries of rat. In these studies, we found a significant correlation between the steady state levels of

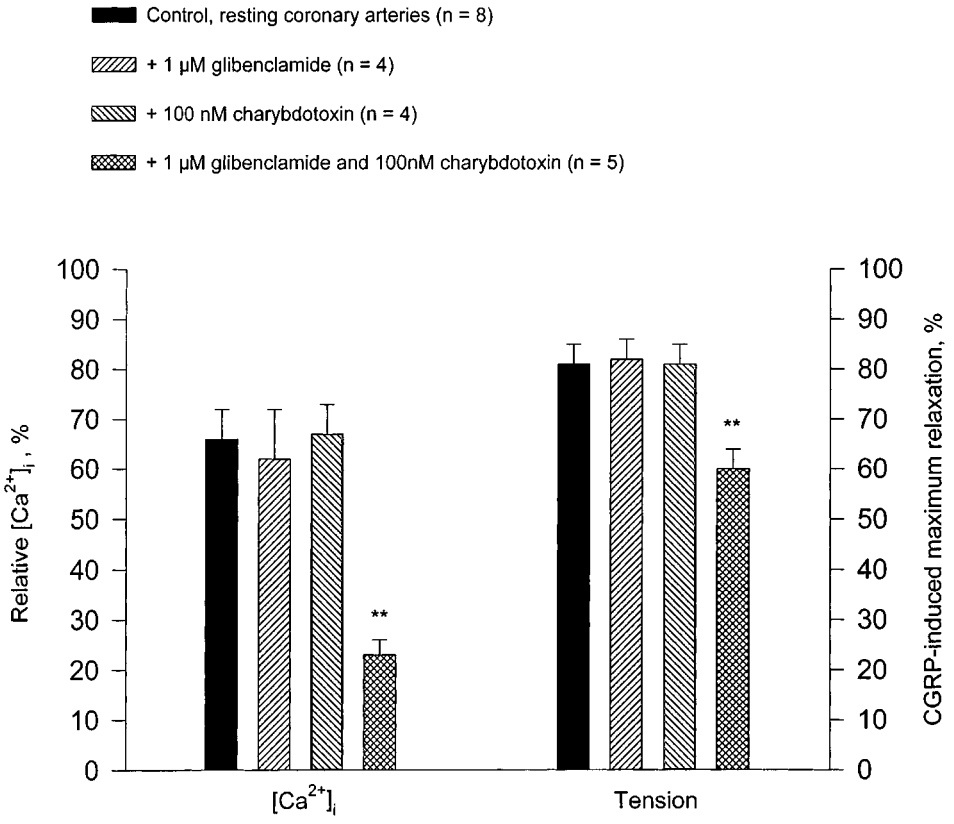

Figure 9 The effect of pretreatment with the combination of 100 nM charybdotoxin and 1 μM glibenclamide on the rat-αCGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of resting coronary arteries kept in PSS. Relative responses are given as percentages of the initial steady-state resting levels of $[Ca^{2+}]_i$ and tension just before the vessels were challenged with rat-αCGRP. Points represent mean values and vertical bars indicate±s.e.mean where this value exceeds the size of symbol. A paired two-tailed t-test was used to compare the mean values (**$P<0.01$).

$[Ca^{2+}]_i$ and the extent of CGRP-induced reduction in $[Ca^{2+}]_i$. The higher the initial steady-state $[Ca^{2+}]_i$ level, the larger is the reduction in $[Ca^{2+}]_i$ by CGRP. Moreover, these results (see Figure 2) show that the extent of CGRP-induced reduction in $[Ca^{2+}]_i$ depends upon the initial experimental conditions such as resting condition, precontraction and the kind of stimulus used for precontraction. However, when rat coronary arteries were depolarized with high K^+ (36 mM K^+) CGRP only reduced the tension without significantly affecting the steady state $[Ca^{2+}]_i$ level induced by 36 mM K^+. Furthermore, our results from experiments with BAY K 8644 (prolongs opening time of voltage-dependent L-type Ca^{2+} channels) and felodipine (blocks the voltage-dependent L-type Ca^{2+} channels) suggest that CGRP may inhibit calcium influx through voltage-dependent L-type Ca^{2+} channels. All these results suggest that CGRP-induced vasorelaxation consists of two components: one which is dependent on the membrane potential or $[Ca^{2+}]_i$ and is inhibited by high K^+-induced depolarization, and another which is not dependent on the membrane potential or $[Ca^{2+}]_i$ and is resistant to high K^+-induced depolarization.

There is an agreement between our results in rat coronary arteries and the previous findings in porcine coronary arteries using FURA-2 technique (Fukuizumi *et al*., 1996). In these studies, authors demonstrated an inhibition of Ca^{2+} influx by CGRP in histamine-precontracted porcine coronary arteries. The authors also showed that CGRP reduced the tension during depolarization with high K^+ (30–118 mM), without affecting the $[Ca^{2+}]_i$ levels. In addition, they were able to demonstrate that the inhibition by CGRP of the depolarization-induced Ca^{2+}-influx was dependent upon the concentration of extracellular potassium ($[K^+]_o$) since CGRP only decreased the $[Ca^{2+}]_i$ of coronary arteries weakly depolarized with $[K^+]_o$ lower than 20 mM (Fukuizumi *et al*., 1996). Such a $[K^+]_o$-dependence has also been observed in the inhibition of Ca^{2+} influx by isoprenaline that elevates cyclic AMP levels (Ushi-Fukai *et al*., 1993). A number of studies have shown that CGRP increases the cytosolic cyclic AMP levels in various kinds of vascular smooth muscles, including the coronary arteries (Shoji *et al*., 1987; Gray & Marshall, 1991; Yoshimoto *et al*., 1998; Wisskirchen *et al*., 1999). Furthermore, CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension were mediated by an increase in smooth muscle cyclic AMP content, because these effects were potentiated by a phosphodiestrase inhibitor and blocked by a protein kinase A inhibitor (Kageyama *et al*., 1993; Ishikawa *et al*., 1993).

We have previously shown that CGRP induces an endothelium-independent relaxation in rat intramural coronary arteries, indicating a direct action of CGRP on vascular smooth muscles (Prieto *et al*., 1991; Sheykhzade & Nyborg, 1998). Our recent studies show that CGRP exerts its direct vasodilatory action by increasing the cyclic AMP levels, thereby activating the cyclic AMP-PKA pathway in rat intramural coronary arteries (data not shown). It has been reported that cyclic AMP hyperpolarizes the arterial smooth muscle in low concentrations of $[K^+]_o$ but does not have a significant effect on the membrane potential in the presence of high concentration of $[K^+]_o$ (Somlyo *et al*., 1970) and that CGRP also produces the hyperpolarization of vascular smooth muscle by activating K^+ channels (Nelson *et al*., 1990; Kitazono *et al*., 1993). It is likely that the one component that is sensitive to high K^+-depolarization may be mediated by the membrane hyperpolarization. This notion is supported by the previous observation that activation of K^+ channels by K^+ channel openers is counteracted by increases in $[K^+]_o$ (Hamilton *et al*., 1986). K^+ channel openers fail to relax K^+-depolarized muscle when the extracellular K^+ concentration moves the K^+ equilibrium potential beyond that needed for activation of the Ca^{2+} channels. Therefore, our results support the idea that CGRP may inhibit the Ca^{2+} influx, at least in part, through membrane hyperpolarization. However, further studies on the membrane potential are still needed to determine the involvement of membrane hyperpolarization in the CGRP-induced reduction of $[Ca^{2+}]_i$ in coronary artery smooth muscle.

Several studies have shown that cyclic AMP stimulates Ca^{2+} uptake into the sarcoplasmic reticulum (SR) through activation of Ca^{2+}- pump ATPase (Raeymaekers *et al*., 1990; Kimura *et al*., 1982). Previous studies on porcine coronary arteries using the FURA-2 technique, revealed that isoprenaline, a β-adrenoceptor agonist which is well known to increase cytosolic cyclic AMP, similarly stimulates the uptake of Ca^{2+} into the ryanodine-sensitive storage sites (Ushio-Fukai *et al*., 1993). Another study carried out by Kawasaki *et al*. (1997) on porcine coronary arteries demonstrated that vasoactive intestinal peptide, which also elevates the cytosolic cyclic AMP, partly decreased $[Ca^{2+}]_i$ by sequestrating cytosolic Ca^{2+} into the sarcoplasmic reticulum (SR). Therefore, it was hypothesized that the CGRP-induced decrease in $[Ca^{2+}]_i$ in rat coronary arteries could partly be mediated by the uptake of cytosolic Ca^{2+} into the intracellular storage sites. In our studies, pretreatment with thapsigargin, a selective blocker of the sarcoplasmic reticulum Ca^{2+}- pump ATPase (SERCA) caused a significant attenuation of the CGRP-induced decrease in the tension. However, the CGRP-induced decrease in $[Ca^{2+}]_i$ was not significantly attenuated by this treatment. It could be speculated that slight changes in $[Ca^{2+}]_i$ at the contractile proteins is enough to cause the observed changes in the tension.

Another component of CGRP-induced vasorelaxation could be explained by a decrease in Ca^{2+}sensitivity of the contractile apparatus. It is generally accepted that phosphorylation of 20-kDa myosin p-light chain (MLC_{20}) by a Ca^{2+}- and calmodulin-dependent enzyme, myosin light chain kinase (MLCK), plays an important role in the regulation of smooth muscle contraction (Kamm & Stull, 1985; Somlyo & Somlyo, 1994). However, the smooth muscle contraction is also regulated by the action of phosphatases (see Somlyo *et al*., 1999 for review). These studies indicate that the balance between the activity of MLCK and MLC phosphatase is the major direct controller of contraction in the smooth muscle cells. *In vitro* studies indicate that cyclic nucleotides (e.g. cyclic AMP) can cause decreased Ca^{2+}sensitivity either by phosphorylating the myosin light chain kinase (Adelstein *et al*., 1978; Hathaway *et al*., 1985) or by increasing the activity of the MLC phosphatases (Wu *et al*., 1996; Van Riper *et al*., 1997). It was previously shown that cyclic AMP and the catalytic subunits of protein kinase A were able to relax membrane-permeabilized smooth muscles, contracted with constant $[Ca^{2+}]_i$ (Kerrik & Hoar, 1981; Itoh *et al*., 1982; Nishimura & Van Breemen, 1989).

However, further investigations are required to determine the possible role of MLCK-phosphorylation, phosphatases

and cyclic AMP-PKA pathway in CGRP-induced decrease in Ca^{2+} sensitivity of contractile apparatus in rat coronary arteries.

The role of K^+ channels in the CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of U46619-precontacted rat coronary arteries

One of the novel findings of the present study is that the CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of precontracted coronary arteries was significantly attenuated by either charybdotoxin or iberiotoxin, indicating that large conductance Ca^{2+}-activated K^+ channels (K_{Ca}) may contribute to the CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension. However, charybdotoxin was more effective in attenuating the CGRP-induced responses in rat coronary arteries compared to iberiotoxin. This could be explained by toxins having different kinetics or selectivities for K^+ channels. It has been reported that charybdotoxin, in some preparations (mostly in cell cultures), can also block the slowly inactivating voltage-gated K^+ channels (Kv 1.3), whereas it does not block the voltage-gated K^+ channels of freshly isolated smooth muscle cells (see Beech, 1997 for review). Therefore, there is a possibility that CGRP may exert its action in rat intramural coronary arteries by activating more than one type of K^+ channels, which requires further investigation. In our study, ATP-sensitive K^+ channels (K_{ATP}) were not involved in the CGRP-induced responses when the smooth muscle was activated by U46619. This is in agreement with the results from our previous study on isolated intramural coronary arteries from Wistar rats (Prieto *et al*., 1991), consistent with the findings in isolated porcine coronary arteries (Kageyama *et al*., 1993).

Patch-clamp and electrophysiological experiments have shown that CGRP can hyperpolarize vascular smooth muscle cell membrane by opening K_{ATP} and K_{Ca} channels (Nelson *et al*., 1990; Kitazono *et al*., 1993; Miyoshi & Nakaya, 1995; Hong *et al*., 1996; Standen & Quayle, 1998). Furthermore, it was shown that CGRP activated these potassium channels *via* cyclic AMP-PKA pathway (Quayle *et al*., 1994; Miyoshi & Nakaya, 1995; Wellman *et al*., 1998). However, the involvement of potassium channels in CGRP-induced vasodilatation is not only dependent upon the kind of tissue used in experiments, but also on the experimental conditions. CGRP mediates vasodilatation through K_{ATP} channels in human internal mammary arteries but not in gastroepiploic arteries (Luu *et al*., 1997). Furthermore, conflicting results have been observed comparing cultured systems (Miyoshi and Nakaya, 1995; Wellman *et al*., 1998) with more complex isolated tissues (Prieto *et al*., 1991; Kageyama *et al*., 1993). Functional studies are usually performed on precontracted vessels and the agents used for precontraction can activate protein kinase C (PKC). A number of studies have shown an inhibition of both K_{ATP} channels (Bonev & Nelson, 1993; Beech, 1997; Standen & Quayle, 1998) and K_{Ca} channels (Minami *et al*., 1993, 1995; Beech, 1997; Standen & Quayle, 1998) through PKC. Therefore, in the present study we also investigated the effects of selective blockers of these channels on CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of resting coronary arteries in PSS.

The role of K^+ channels in the CGRP-induced decrease in the $[Ca^{2+}]_i$ and tension of resting coronary arteries in PSS

Another novel finding of the present study is that neither glibenclamide nor charybdotoxin alone were able to attenuate the CGRP-induced decrease in the $[Ca^{2+}]_i$ or tension of resting coronary arteries kept in PSS. Only when glibenclamide and charybdotoxin were added together, was the CGRP-induced decrease in the resting $[Ca^{2+}]_i$ and basal tension significantly attenuated.

It is well known that the function of ion channels can be affected by the membrane potential and/or by different coupling mechanisms (second messengers). K_{Ca} channels serve as an important negative feedback mechanism that indirectly sets the level of Ca^{2+} entry through voltage-dependent L-type Ca^{2+} channels by shifting membrane potential in a direction (near the resting membrane potential) that reduces their steady-state open probability (Guia *et al*., 1999). However, K_{Ca} channel activity is not only regulated by Ca^{2+} entry through L-type Ca^{2+} channels, but also by Ca^{2+} release from the SR (Singer & Walsh, 1986; see also Beech, 1997 for review). The release of Ca^{2+} from the SR by a chemical messenger, inositol 1,4,5-triphosphate (IP_3), is well known to be initiated not by depolarization, but by agonist-receptor interaction (e.g. during contraction by receptor-dependent agonists such as U46619) (see Somlyo *et al*., 1999 for review). All these studies indicate that the activation of K^+ channels by CGRP can be dependent on the choice of initial experimental conditions under which the vessel is placed.

In conclusion, CGRP relaxes the precontracted rat coronary arteries by decreasing not only the $[Ca^{2+}]_i$ but also the Ca^{2+} sensitivity of the contractile apparatus. It seems that CGRP may decrease $[Ca^{2+}]_i$ mainly by inhibiting the Ca^{2+} influx through membrane hyperpolarization induced partly by activation of K_{Ca} channels. In our study, we can not rule out the possibility that CGRP may cause a slight (but not significant) decrease in the $[Ca^{2+}]_i$ by sequestrating cytosolic Ca^{2+} into thapsigargin-sensitive Ca^{2+} storage sites. In resting coronary arteries, however, there seems to be an interplay between different types of K^+ channels, suggesting a different mechanism of action for CGRP in precontracted- and resting coronary arteries.

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