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European Journal of Pharmacology 484 (2004) 91–101
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Homologous desensitization of calcitonin gene-related peptide-induced relaxation in rat intramural coronary arteries

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Received 7 August 2003; received in revised form 23 October 2003; accepted 31 October 2003

Abstract

We investigated the type of desensitization of calcitonin gene-related peptide (CGRP)-induced responses in rat isolated intramural coronary arteries using isometric myograph and FURA-2 technique. In coronary arteries precontracted with 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619), development of tachyphylaxis to CGRP is characterized by significant attenuation of CGRP-induced maximal reduction in the tension and $[Ca^{2+}]_i$ during the second CGRP concentration–response curve; however, there was no further reduction in the CGRP-induced maximum relaxation during the third CGRP concentration–response curve. There was no sign of tachyphylaxis to CGRP when CGRP concentration–response curves were recorded in 36 mM K^+-depolarized coronary arteries contrary to the results obtained in 300 nM U46619-precontracted coronary arteries. Preincubation with colchicine did not prevent the development of tachyphylaxis to CGRP in U46619-precontracted coronary arteries, indicating no role for endocytosis. Development of tachyphylaxis to CGRP was completely abolished by preincubating the coronary arteries with 1 μM RO 31-8220, indicating a role for protein kinases. Pre-exposure of the coronary arteries to isoprenaline or forskolin did not attenuate the CGRP-induced relaxation in these vessels, indicating that the cAMP–protein kinase A (PKA) pathway is not involved. Like CGRP, the coronary arteries developed tachyphylaxis toward isoprenaline during the second exposure. However, there was no sign of tachyphylaxis to either forskolin or dibutyryl cAMP (dbcAMP) during the second exposure. In conclusion, these results suggest that development of tachyphylaxis to CGRP in U46619-precontracted coronary is related to CGRP receptor-mediated activation of protein kinase.

Keywords: Desensitization; CGRP (calcitonin gene-related peptide); Coronary artery

1. Introduction

It is well known that G-protein-coupled receptors (e.g. adrenoceptors) are rapidly desensitized (Sibley and Lefkowitz, 1985; January et al., 1998). The sequence of the G-protein-coupled receptors includes certain residues (serine and threonine) mainly in the C-terminal cytoplasmic tail, which act as phosphorylation sites where specific or nonspecific kinase enzymes catalyse the coupling of phosphate groups. The key difference between receptor desensitization by G-protein-coupled receptor kinases (GRKs: 1, 2, 3, 4, 5 and 6) and protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) is that GRKs specifically phosphorylate agonist-activated G-protein-coupled receptors (homologous desensitization) whereas the kinases involved in heterologous desensitization (e.g. PKA and PKC) may phosphorylate either the receptor that was just activated to stimulate it, or additional receptors in a promiscuous fashion (see Chuang et al., 1996 for review).

Phosphorylated receptors are bound by arrestin, which precludes receptor–G protein interaction leading to functional desensitization. The ligand–receptor complex is then removed from plasma membrane and internalized via clathrin into vesicles that soon shed their clathrin coat and become early endosomes (endocytosis). Finally, the internalized receptors can recycle, thereby contributing to resensitization of cellular responses or be degraded by peroxisomes (see Koenig and Edwardson, 1997; Pitcher et al., 1998; Ferguson, 2001 for review).

Calcitonin gene-related peptide (CGRP) is a naturally occurring 37-amino-acid peptide that is found throughout the central and peripheral nervous system. In vitro studies

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doi:10.1016/j.ejphar.2003.10.052

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 and 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 and Lundberg, 1985; Franco-cereceda and Liska, 2000) and in vivo (Kallner, 1998) during hypoxia and by low pH levels in the myocardium, thus suggesting a vasodilatory role under ischemic conditions.

CGRP receptors belong to the family of G-protein-coupled receptors characterized by seven transmembrane helices (Chatterjee et al., 1991, 1993; Chatterjee and Fisher, 1995). CGRP receptors are subdivided into two types, designated $CGRP_1$ and $CGRP_2$ receptors (Quirion et al., 1992; Poyner, 1995). We have previously shown that CGRP-induced endothelium-independent relaxation occurs through activation of $CGRP_1$-receptor subtypes in rat intramural coronary arteries (Sheykhzade and Nyborg, 1998a).

Tachyphylaxis or attenuation of the vascular response to calcitonin gene-related peptide (CGRP) has also previously been shown in many vascular preparations including rat (Prieto et al., 1991; Sheykhzade and Nyborg, 1998b) and porcine (Gray and Marshall, 1991; Marshall, 1992) coronary arteries. We have previously demonstrated that pre-exposure of rat coronary arteries (precontracted with prostaglandin $F_{2\alpha}$) to CGRP resulted in attenuation of CGRP-mediated relaxation by ≈45% without affecting the sensitivity of coronary arteries to CGRP (Sheykhzade and Nyborg, 1998b). Furthermore, we showed that intermittent depolarization of coronary arteries during the washout period with the buffer solution containing 125 mM K^+ (KPSS) completely blocked the development of tachyphylaxis in these vessels (Sheykhzade and Nyborg, 1998b). In general, little is known regarding the detailed mechanism behind the desensitization of CGRP receptors. However, recent studies performed on human embryonic kidney (HEK-293) cells have shown that calcitonin receptor-like receptor (CRL receptor) and receptor activity-modifying proteins (RAMPs) were internalized (endocytosed) together via clathrin-coated vesicles following ligand exposure, and both the internalized molecules were targeted to the degradative pathway (Kuwasako et al., 2000; Hilairet et al., 2001). Furthermore, it was shown in HEK-293 cells that G-protein receptor kinase 6 was involved in the CGPR-induced desensitization (Aiyar et al., 2000). It is worth mentioning that all these studies were performed on HEK cells, which do not necessarily represent the signal transduction and effector proteins found in smooth muscle cells of intact arteries.

The purpose of our study was to investigate the type of desensitization toward CGRP-induced responses in isolated rat intramural coronary arteries. This investigation will shed light on the complexity of the signal transduction involved in CGRP-induced desensitization in isolated arteries.

2. Materials and methods

2.1. Tissue preparation

All animal procedures were strictly within national laws and guidelines. Three-month-old male Sprague Dawley rats were stunned by blow on the head prior to exsanguinations (decapitation). Then, the heart was rapidly removed and arterial ring segments (1–2 mm long, one arterial segment per rat) were isolated from the same anatomical location in the distal, intramural, part of the left coronary artery as previously described (Nyborg et al., 1987).

2.2. Measurement of force development

The coronary arteries were mounted on an isometric myograph (Danish Myo Technology, Aarhus, Denmark) as previously described (Mulvany and Nyborg, 1980). After mounting, the arteries were equilibrated in oxygenated (5% CO_2 in O_2) physiological salt solution (PSS) at 37 °C, pH 7.4, for 30 min. The vessels were then stretched to an internal circumference, L_1, equal to 90% of the circumference, L_{100}, the vessel would have if exposed to a passive transmural pressure of 100 mm Hg (13.3 kPa) (Nyborg et al., 1987) in order to secure maximal active force development. The effective vessel lumen diameter was calculated as L_1/π.

The vessels were contracted at least thrice with the buffer solution containing 125 mM K^+ (KPSS, similar to PSS except that NaCl was exchanged for KCl on an equimolar basis) until reproducible contractions were recorded. The maximal contractile response of the vessels (ΔT_{max}) was determined at the end of each experiment by measuring the difference in vessel wall tension (Newton per meter of vessel wall, N m^{-1}) when the vessels were maximally contracted with supra activating cocktail solution (KPSS to which 10 μM prostaglandin $F_{2\alpha}$ and serotonin were added) and when maximally relaxed in Ca^{2+}-free PSS (similar 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)).

2.3. FURA-2 loading procedure and measurement of $[Ca^{2+}]_i$

The coronary arteries were loaded with the fluorescent $[Ca^{2+}]_i$ indicator dye (FURA-2) using exactly the same procedure as previously described (Sheykhzade and Nyborg, 2001). All experiments with FURA-2 were performed in the dark. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the equation; $[Ca^{2+}]_i = K_d\beta[(R - R_{min})/(R_{max} - R)]$, with the assumption that the dissociation con-

stant of FURA-2–Ca^{2+} complex, K_d, is 224 nM at 37 °C (Grynkiewicz et al., 1985). The parameters β, R_{min} and R_{max} (all corrected for background fluorescence signals) were determined in each vessel at the end of the experiment using exactly the same procedure as previously described (Sheykhzade and Nyborg, 2001). The mean values ($n=11$) of R_{max}, R_{min} and β were 3.98 ± 0.05, 1.24 ± 0.03 and 1.74 ± 0.15, 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 tension, respectively.

2.4. Drugs and solutions

PSS had the following composition (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.5, K_2HPO_4 1.18, $MgSO_4$ 1.17, ethylene diamine tetraacetic acid (EDTA) 0.026 and glucose 5.5, pH 7.4. KPSS (125 mM K^+) was prepared by replacing NaCl with equimolar KCl.

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 prostaglandin $F_{2\alpha}$ (Dinoprost®, Upjohn, Puurs, Belgium), U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) (Fluka Chemie, Switzerland), ionomycin, pluronic F127, cremophor EL, $MnCl_2$, rat-αCGRP, 5-hydroxytryptamine HCl, Dibutyryl cAMP (dbcAMP) (Sigma, St. Louis, MO, USA) and FURA-2 acetylmethyl ester (FURA-2AM) (Molecular Probes, Leiden, The Netherlands). RO 31-8220 (2-{1-[3-(Amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide methanesulfonate) was kindly provided by Roche Discovery (Welwyn, UK). RO 31-8220 was dissolved in distilled water just before use. FURA-2AM was dissolved in loading mixture (anhydrous dimethylsulphoxide (DMSO), pluronic F-127 and cremophor EL) just before loading. U46619 was dissolved in 50% ethanol at 10^{-2} M. Rat-αCGRP was dissolved in distilled water and stored frozen at −20 °C until use. Dilutions of the stock solutions were made fresh each day.

2.5. Data analysis and statistics

Relaxations are expressed as a percentage of U46619-induced tension (precontraction tension). The precontraction tensions induced by U46619 or 35 mM K^+ (measured as N m^{-1}) are expressed as percentage of the steady-state contractile response to the cocktail solution (percentage of ΔT_{max}). The levels of $[Ca^{2+}]_i$ (measured as nM) are given as percentage of the plateau levels.

The concentration–response curves to CGRP were fitted to the classical 'Hill equation': $E/E_{max}=[A]^n/([A]^n + EC_{50}[M]^n)$ using the GraphPad Prism 3.0 software. E/E_{max} is the relative vessel response to the agonist concentration, [A]. EC_{50}[M] is concentration of agonist required to give half-maximal response, and n is a fitting constant or 'Hill coefficient' (Kenakin, 1997). Sensitivity to CGRP and other vasoactive agents is expressed in terms of pD_2 values, where $pD_2 = -\log(EC_{50}[M])$. The tachyphylaxis was calculated as relative reduction (%) in the CGRP-induced maximum relaxation comparing the first and second concentration–response curves. Results are given as mean± S.E.M. (n = number of rats). Differences between mean values were analysed by a two-tailed Student's t-test for paired or unpaired where appropriate. Results were considered to be significant if P value <0.05.

3. Results

3.1. CGRP-induced reduction in $[Ca^{2+}]_i$ and tension during tachyphylaxis

In the first series of experiments, we investigated the ability of CGRP to reduce both the tension and $[Ca^{2+}]_i$ in isolated rat coronary arteries upon development of tachyphylaxis. Two consecutive cumulative concentration–response curves with CGRP were recorded in 300 nM U46619-precontracted coronary arteries with or without activation with KPSS buffer during the washout period between the concentration–response curves as previously described (Sheykhzade and Nyborg, 1998b). There was a significant ($P<0.01$) attenuation of CGRP-induced maximal reduction in the $[Ca^{2+}]_i$ and tension during the second CGRP concentration–response curves in the experiments where coronary arteries were only washed with PSS buffer during the washout period (Fig. 1; Table 1). The mean lumen diameter of vessels used was 207 ± 5 μm ($n=5$).

When coronary arteries were depolarized with KPSS (125 mM K^+) during the washout period, there was no significant difference in CGRP-induced maximal reduction in the $[Ca^{2+}]_i$ or tension between the first and second CGRP concentration–response curves (Fig. 1; Table 1). The mean lumen diameter of vessels used was 200 ± 9 μm ($n=6$).

3.2. Three consecutive concentration–response curves with CGRP

In the second series of experiments, three consecutive cumulative concentration–response curves with CGRP (10 pM–100 nM) were recorded in 300 nM U46619-precontracted coronary arteries with a 15-min washout period where coronary arteries were only washed with PSS buffer.

The maximal relaxation induced by CGRP was as usual significantly ($P<0.01$) reduced during the second CGRP concentration–response curve as compared to the first CGRP concentration–response curve, but the sensitivity of coronary arteries to CGRP was similar (Fig. 2; Table 1). During the third CGRP concentration–response curve, however, there was no further decrease in the maximal relaxation induced by CGRP. The CGRP-induced maximal relaxation in the third

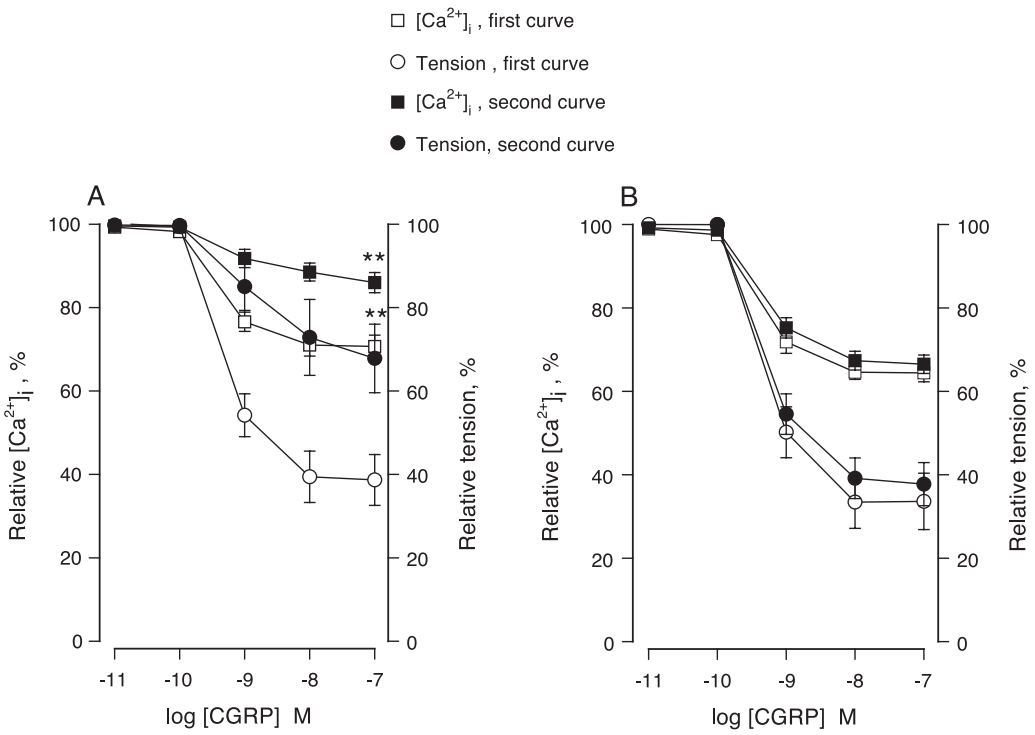

Fig. 1. The reproducibility of CGRP-induced reduction in the $[Ca^{2+}]_i$ and tension of 300 nM U46619-precontracted coronary arteries either washed with (A) PSS buffer ($n=5$) or activated with (B) KPSS buffer containing 125 mM K^+ ($n=6$) during the 15-min washout period. Points represent mean values and vertical bars indicate ± S.E.M. Relative tension and $[Ca^{2+}]_i$ are given as percentages of the initial steady-state levels induced by U46619 (300 nM). A paired two-tailed *t*-test was used to compare the mean values between the first and second curves (**$P<0.01$).

concentration–response curve was similar to that in the second concentration–response curve and the sensitivity of coronary artery to CGRP in the third concentration–response curve was similar to those observed in the first and second CGRP concentration–response curves (Fig. 2). The mean lumen diameter of vessels used was 208 ± 14 μm ($n=6$).

Table 1
Reproducibility of CGRP concentration–response curves in coronary arteries precontracted with either 300 nM U46619 or 36 mM K^+

Experimental protocols	*n*	First curves	Second curves	Third curves	Tachyphylaxis, %	*P*
Two CGRP curves, U46619						
Maximum relaxation with CGRP (+KPSS), %	6	66 ± 7	62 ± 5	–	6 ± 4	NS
Maximum relaxation with CGRP (−KPSS), %	5	61 ± 6	33 ± 8[a]	–	46 ± 7	0.0053
Precontraction tone (+KPSS), % of ΔT_{max}	6	74 ± 3	72 ± 4			NS
Precontraction tone (−KPSS), % of ΔT_{max}	5	70 ± 5	69 ± 2			NS
pD_2 for CGRP (+KPSS)	6	9.16 ± 0.05	9.14 ± 0.06	–	–	NS
pD_2 for CGRP (−KPSS)	5	9.17 ± 0.06	9.08 ± 0.07	–	–	NS
Maximum reduction in $[Ca^{2+}]_i$ (+KPSS), %	6	35 ± 2	33 ± 2	–	–	NS
Maximum reduction in $[Ca^{2+}]_i$ (−KPSS), %	5	30 ± 3	14 ± 2[a]	–	–	0.0080
Three CGRP curves, U46619						
Maximum relaxation with CGRP (−KPSS), %	6	81 ± 9	49 ± 10[a]	48 ± 9[a]	40 ± 7	<0.01
Precontraction tone (−KPSS), % of ΔT_{max}	6	71 ± 3	69 ± 4	71 ± 4	–	NS
pD_2 for CGRP (−KPSS)	6	8.96 ± 0.08	8.86 ± 0.08	8.82 ± 0.07	–	NS
Two CGRP curves, 36 mM K^+						
Maximum relaxation with CGRP (−KPSS), %	6	45 ± 7	44 ± 7	–	2 ± 8	NS
Precontraction tone (−KPSS), % of ΔT_{max}	6	68 ± 4	61 ± 5	–	–	NS
pD_2 for CGRP (−KPSS)	6	8.90 ± 0.07	8.89 ± 0.06	–	–	NS

+KPSS and −KPSS indicate with and without KPSS activation during the washout period, respectively. Values are given as mean ± S.E.M. A paired two-tailed *t*-test was used for comparison of the mean values.
NS = not significant.
[a] $P<0.01$ vs. first concentration–response curves.

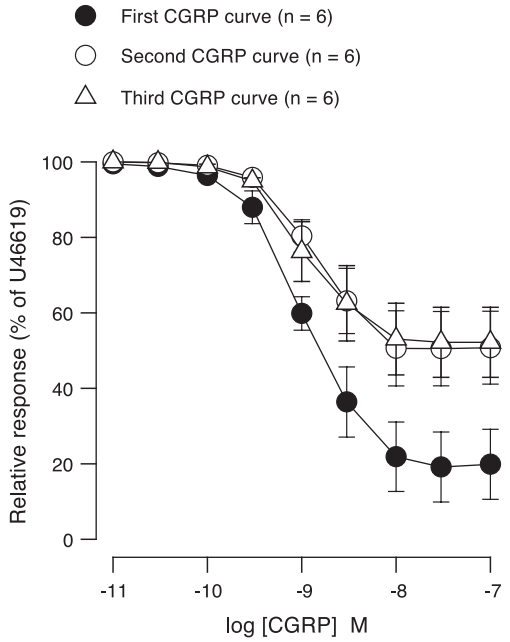

Fig. 2. Three consecutive cumulative concentration–response curves with CGRP (10 pM–100 nM) recorded in 300 nM U46619-precontracted coronary arteries. Responses are given as percentages of the initial steady-state response to U46619 (300 nM) just before the vessels were challenged with CGRP. Points represent mean values and vertical bars show ± S.E.M., where this exceeds the size of the symbol.

3.3. Reproducibility of CGRP concentration–response curves in 36 mM K^+-depolarized arteries

In the third series of experiments, two consecutive cumulative concentration–response curves with CGRP (10 pM–100 nM) were recorded in 36 mM K^+-depolarized coronary arteries with a 15-min washout period where coronary arteries were only washed with PSS buffer. There was no indication of tachyphylaxis to CGRP when concentration–response curves were recorded in 36 mM K^+-depolarized coronary arteries (Fig. 3; Table 1) in contrast to the results obtained in U46619-precontracted coronary arteries. The mean lumen diameter of vessels used was 210 ± 17 μm ($n = 6$).

3.4. Effect of colchicine on the reproducibility of CGRP concentration–response curves

In the fourth series of experiments, the effect of colchicine (an inhibitor of tubulin polymerization) on the CGRP-induced tachyphylaxis was investigated. The coronary arteries were preincubated with 10 μM colchicine for 15 min and then two consecutive cumulative concentration–response curves with CGRP (10 pM–100 nM) were recorded in 300 nM U46619-precontracted vessels with a 15-min washout period in the presence of colchicine. Incubation with colchicine did not affect the resting tone or precontration tone of rat coronary arteries. Incubation of coronary arteries with 10 μM colchicine had no significant effect on the development of tachyphylaxis to CGRP in these vessels (Fig. 4A; Table 2). Both potency (pD_2) and efficacy of CGRP were significantly ($P < 0.05$) reduced during the second CGRP concentration–response curve. The mean lumen diameter of vessels used was 199 ± 10 μm ($n = 6$).

3.5. Effect of RO 31-8220 on the reproducibility of CGRP concentration–response curves

In the fifth series of experiments, the role of protein kinases in the development of tachyphylaxis to CGRP was investigated by using 1 μM RO 31-8220, a nonspecific inhibitor of PKC and also of other protein kinases (Alessi, 1997; Kutz et al., 1998; Aiyar et al., 2000; Davies et al., 2000). After recording the first concentration–response curve for CGRP, the coronary arteries were incubated with 1 μM RO 31-8220 for 30 min. After 30 min of incubation, the inhibitor was removed from the system by thorough washing before constructing the second concentration–response curve for CGRP. Pretreatment with 1 μM RO 31-8220 completely abolished the development of tachyphylaxis to CGRP (Fig. 4B; Table 2). There was no significant difference in either maximum relaxation induced by CGRP or sensitivity to CGRP between the first and second CGRP concentration–response curves. The mean effective lumen diameter of vessels used was 215 ± 20 μm ($n = 6$).

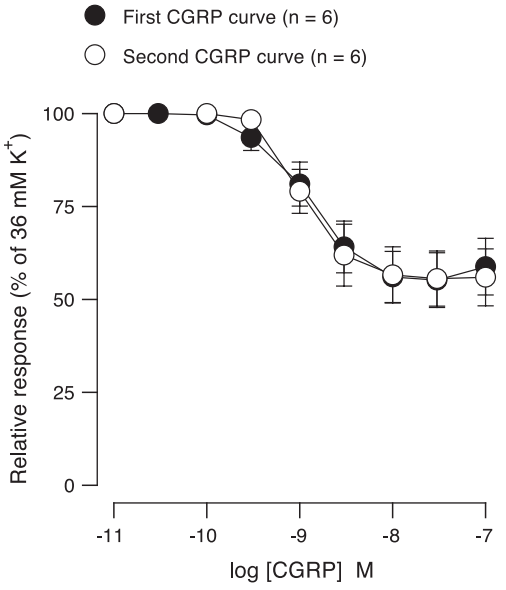

Fig. 3. The reproducibility of CGRP concentration–response curves (10 pM–100 nM) recorded in 36 mM K^+-depolarized coronary arteries. Responses are given as percentages of the initial steady-state response to 36 mM K^+ just before the vessels were challenged with CGRP. Points represent mean values and vertical bars show ± S.E.M., where this exceeds the size of the symbol.

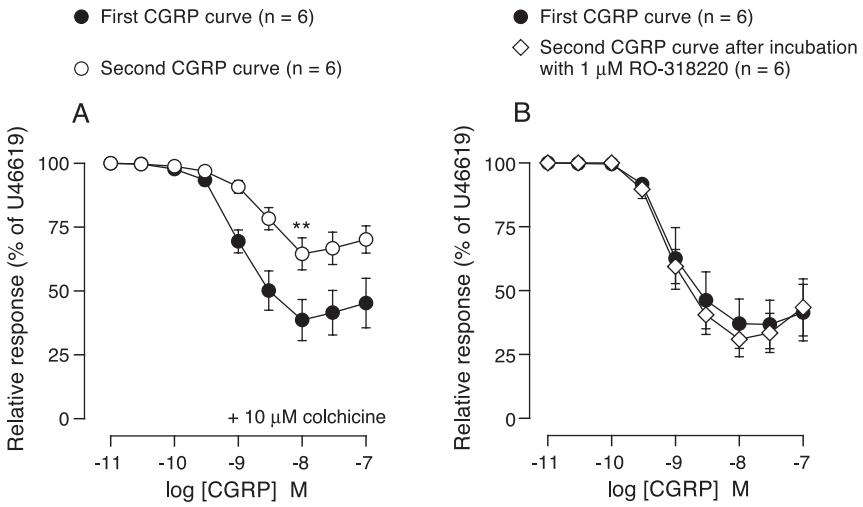

Fig. 4. The effect of (A) 10 μM colchicine and (B) 1 μM RO 31-8220 on the reproducibility of CGRP concentration–response curves (10 pM–100 nM) recorded in 300 nM U46619-precontracted coronary arteries. Responses are given as percentages of the initial steady-state response to U46619 (300 nM). Points represent mean values and vertical bars show ± S.E.M., where this exceeds the size of the symbol. A paired two-tailed *t*-test was used to compare the mean values between the first and second curves (**$P < 0.01$).

3.6. Effect of cAMP–PKA activation on the reproducibility of CGRP concentration–response curves

In the sixth series of experiments, we investigated whether activation of cAMP–PKA pathway (cross-desensitization) was involved in the development of tachyphylaxis to CGRP in intramural coronary arteries by using isoprenaline and forskolin. Three consecutive cumulative concentration–response curves were constructed in 300 nM U46619-precontracted coronary arteries. The first concentration–response curve was made with CGRP (10 pM–100 nM) and then coronary arteries were activated twice with the KPSS buffer (125 mM K^+) during the washout period in order to overcome development of tachyphylaxis to CGRP. The second concentration–response curve was made with either isoprenaline or for-

Table 2
Effect of pretreatment with colchicine, RO 31-8220, forskolin and isoprenaline on the reproducibility of CGRP concentration–response curves in U46619-precontracted coronary arteries

Experimental protocols	*n*	First CRCs	Second CRCs	Tachyphylaxis, %	*P*
Incubation with colchicine (− KPSS)					
Maximum relaxation with CGRP, %	6	63 ± 8	36 ± 6[a]	43 ± 9	0.0092
Precontraction tone, % of ΔT_{max}	6	73 ± 6	76 ± 8	–	NS
pD_2 for CGRP	6	9.06 ± 0.08	8.71 ± 0.06[b]	–	0.0106
Pre-exposure to isoprenaline (− KPSS)					
Maximum relaxation with CGRP, %	8	77 ± 7	72 ± 5	7 ± 4	NS
Precontraction tone, % of ΔT_{max}	8	76 ± 2	75 ± 4	–	NS
pD_2 for CGRP	8	8.82 ± 0.08	8.75 ± 0.06	–	NS
Pre-exposure to forskolin (− KPSS)					
Maximum relaxation with CGRP, %	4	77 ± 5	73 ± 5	5 ± 2	NS
Precontraction tone, % of ΔT_{max}	4	73 ± 2	75 ± 4	–	NS
pD_2 for CGRP	4	8.95 ± 0.04	8.96 ± 0.03	–	NS
Incubation with RO 31-8220 (− KPSS)					
Maximum relaxation with CGRP, %	6	63 ± 10	70 ± 7	− 14 ± 8	NS
Precontraction tone, % of ΔT_{max}	6	73 ± 6	68 ± 5	–	NS
pD_2 for CGRP	6	9.01 ± 0.13	9.12 ± 0.09	–	NS

− KPSS indicates no activation with KPSS during the washout period. Values are given as mean ± S.E.M. A paired two-tailed *t*-test was used for comparison of the mean values.
NS = not significant.
[a] $P < 0.01$ vs. first concentration-response curves.
[b] $P < 0.05$ vs. first concentration-response curves.

skolin (1 nM–10 μM), and finally, the third curve was made with CGRP (10 pM–100 nM) again but without activation with KPSS buffer.

3.6.1. Effect of pre-exposure to isoprenaline

Exposure of coronary arteries to increasing concentrations of isoprenaline had no significant effect on either maximum relaxation induced by CGRP or sensitivity to CGRP when comparing the first and second CGRP concentration–response curves (Fig. 5A; Table 2). The maximal relaxation induced by isoprenaline was 96 ± 3% (n = 8) and the corresponding pD_2 value was 7.53 ± 0.12 (n = 8). The mean effective lumen diameter of vessels used was 193 ± 11 μm (n = 8).

3.6.2. Effect of pre-exposure to forskolin

Exposure of coronary arteries to increasing concentrations of forskolin had no significant effect on either maximum relaxation induced by CGRP or sensitivity to CGRP when comparing the first and second CGRP concentration–response curves (Fig. 5B; Table 2). The maximal relaxation induced by forskolin was 100 ± 0% (n = 4) and the corresponding pD_2 value was 6.38 ± 0.06 (n = 4). The mean effective lumen diameter of vessels used was 202 ± 13 μm (n = 4).

3.7. Reproducibility of concentration–response curves with isoprenaline, forskolin and dbcAMP

In the seventh series of experiments, two consecutive cumulative concentration–response curves with dbcAMP (10 μM–1 mM), forskolin (1 nM–10 μM) and isoprenaline (1 nM–10 μM) were constructed in 300 nM U46619-precontracted coronary arteries with a 15-min washout period between the concentration–response curves. These experiments were performed in order to compare the reproducibility of relaxations induced by these compounds to that of CGRP and to further investigate a possible mechanism for desensitization (e.g. downregulation of adenylate cyclase or activation of phosphodiesterases) without relation to receptor activation (in a nonclassic manner) because forskolin and dbcAMP act downstream of the receptor.

3.7.1. Two consecutive concentration–response curves with isoprenaline

The maximal relaxation induced by isoprenaline was significantly (P < 0.05) reduced during the second isoprenaline concentration–response curve as compared to the first curve, but the sensitivity of coronary arteries to isoprenaline was similar (Fig. 6A). The isoprenaline-induced maximal relaxations being 76 ± 7% and 54 ± 7% (paired t-test, P = 0.0194, n = 6) and the corresponding pD_2 values being 7.75 ± 0.12 and 7.52 ± 0.13 (n = 6) in the first and second isoprenaline concentration–response curves, respectively. The precontraction tone induced by 300 nM U46619 was 76 ± 2% and 68 ± 3% (n = 6) of ΔT_{max}, in the first and second isoprenaline concentration–response curves, respectively. The tachyphylaxis was calculated to 29 ± 7% (n = 6) comparing the first and second isoprenaline concentration–response curves. The mean effective lumen diameter of vessels used was 215 ± 13 μm (n = 6).

3.7.2. Two consecutive concentration–response curves with forskolin

There was no indication of tachyphylaxis to forskolin in 300 nM U46619-precontracted coronary arteries (Fig. 6B). The maximal relaxation induced by forskolin was 100 ± 0% and 100 ± 0% (n = 4) and the corresponding pD_2 values being 6.36 ± 0.07 and 6.37 ± 0.03 (n = 4) in the first and

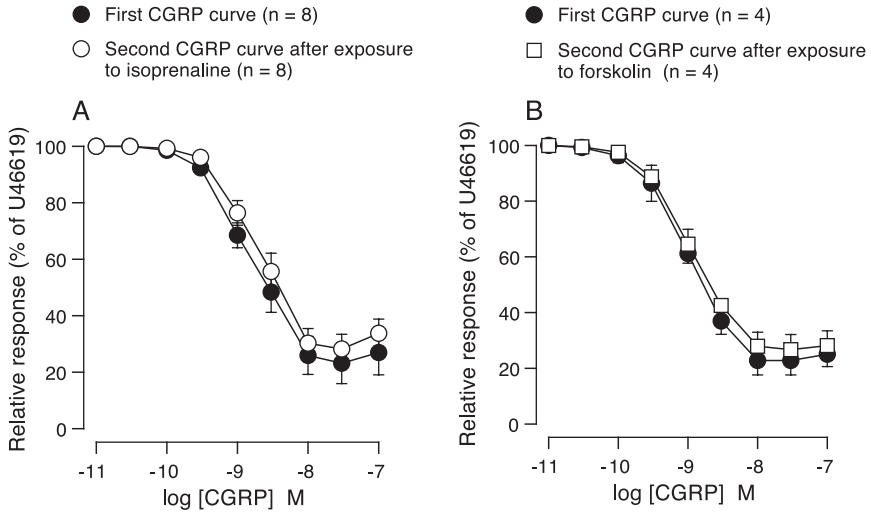

Fig. 5. The effect of pretreatment with (A) isoprenaline (1 nM–10 μM) and (B) forskolin (1 nM–10 μM) on the reproducibility of CGRP concentration–response curves (10 pM–100 nM) recorded in 300 nM U46619-precontracted coronary arteries. Responses are given as percentages of the initial steady-state response to U46619 (300 nM). Points with vertical bars represent mean ± S.E.M.

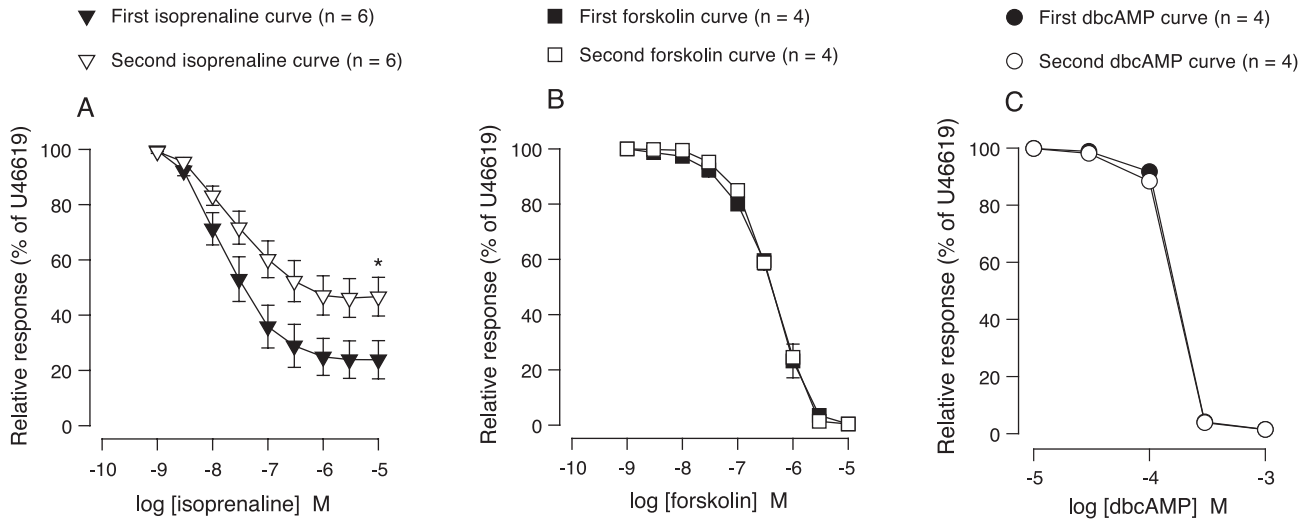

Fig. 6. The reproducibility of concentration–response curves with (A) isoprenaline (1 nM–10 μM), (B) forskolin (1 nM–10 μM) and (C) dbcAMP (10 μM–1 mM) in 300 nM U46619-precontracted coronary arteries. Responses are given as percentages of the initial steady-state response to U46619 (300 nM). Points with vertical bars represent mean ± S.E.M. A paired two-tailed *t*-test was used to compare the mean values between the first and second curves (*$P < 0.05$).

second forskolin concentration–response curves, respectively. The precontraction tone induced by 300 nM U46619 was 75 ± 1% and 79 ± 1% ($n = 4$) of ΔT_{max}, in the first and second forskolin concentration–response curves, respectively. The mean effective lumen diameter of vessels used was 212 ± 6 μm ($n = 4$).

3.7.3. Two consecutive concentration–response curves with dbcAMP

There was no indication of tachyphylaxis to dbcAMP in 300 nM U46619-precontracted coronary arteries (Fig. 6C). The maximal relaxation induced by dbcAMP was 98 ± 1% and 98 ± 1% ($n = 4$) and the corresponding pD_2 values being 3.81 ± 0.01 and 3.83 ± 0.01 ($n = 4$) in the first and second curves, respectively. The precontraction tone induced by 300 nM U46619 was 76 ± 2% and 78 ± 2% ($n = 4$) of ΔT_{max}, in the first and second curves, respectively. The mean effective lumen diameter of vessels used was 214 ± 10 μm ($n = 4$).

4. Discussion

Our present study shows that development of tachyphylaxis to CGRP in U46619-precontracted arteries is characterized by the inability of exogenous CGRP to reduce the tension and $[Ca^{2+}]_i$ of the coronary arteries to the same extent as observed during the first CGRP concentration–response curves. Under these experimental conditions, the CGRP-induced maximum relaxation was reduced by ≈40% during the second CGRP concentration–response curve but the sensitivity of coronary arteries to CGRP was unaffected. However, when coronary arteries were activated with KPSS buffer (containing 125 mM K^+) during the washout period, the CGRP-induced maximal reduction in the $[Ca^{2+}]_i$ and tension were completely restored during the second CGRP concentration–response curve. This is in agreement with our previously study on CGRP-induced tachyphylaxis in prostaglandin $F_{2\alpha}$-precontracted rat intramural coronary arteries (Sheykhzade and Nyborg, 1998b). Studies performed on human embryonic kidney cells and rat glomerular mesangial cell cultures demonstrated that pre-exposure of these cells to CGRP resulted in attenuation of CGRP-mediated cAMP accumulation by 55–60% without affecting the EC_{50} of CGRP for stimulation of cAMP accumulation (Aiyar et al., 1992, 2000).

Strikingly, when three consecutive CGRP concentration–response curves were recorded in U46619-precontracted coronary arteries, there was no further reduction in the CGRP-induced maximum relaxation during the third CGRP concentration–response curves, indicating a limitation or steady-state for development of tachyphylaxis to CGRP in rat coronary arteries. Another finding of the present study was that the development of tachyphylaxis to CGRP was never observed when the CGRP concentration–response curves were recorded in 36 mM K^+-depolarized coronary arteries compared to those experiments performed in prostaglandin $F_{2\alpha}$ (Sheykhzade and Nyborg, 1998b) or U46619-precontracted coronary arteries. A possible explanation is that the depolarization-induced contraction is not a receptor-mediated event, and it is therefore not subject to the G-protein-coupled receptor kinase (GRK)-mediated or other kinase-mediated desensitization seen with U46619 or prostaglandin $F_{2\alpha}$.

Furthermore, the present study showed that the development of tachyphylaxis to CGRP in U46619-precontracted coronary arteries was completely abolished by

preincubating the coronary arteries with RO 31-8220, a nonspecific inhibitor of PKC (Alessi, 1997; Davies et al., 2000), indicating that protein kinases may play role in the development of tachyphylaxis to CGRP in rat intramural coronary arteries.

In most cell culture preparations, CGRP-mediated desensitizations of cAMP accumulation were found to be homologous (Fisher et al., 1988; Aiyar et al., 1992, 2000). There are also situations where cross-desensitization of CGRP receptors have been observed (Drake et al., 2000). In the present study, we showed that the pre-exposure of coronary arteries to isoprenaline (a β-adrenoceptor agonist which is well known to activate cAMP–PKA pathway) or forskolin (a stimulator of adenylate cyclase activity) did not cause attenuation of the CGRP-induced relaxations in these coronary arteries, suggesting that the CGRP-induced desensitization could be homologous in nature and is not related to activation of cAMP–PKA system. Previous studies investigating the reproducibility of CGRP-mediated responses in the porcine cardiovascular system (Franco-cereceda, 1988) and CGRP-induced increase of cAMP production in human embryonic kidney cells (Aiyar et al., 2000) demonstrated that the development of tachyphylaxis to CGRP was not related to downregulation of adenylate cyclase system or activation of PKA or PKC. Instead, Aiyar et al. (2000) by using antisense oligonucleotides for GRK 2, 5 and 6 in human embryonic kidney cells expressing CGRP receptors demonstrated that G-protein receptor kinase 6 (GRK-6) was involved in the desensitization process for CGRP receptors. Furthermore, Franco-cereceda (1991) showed that the tachyphylaxis to CGRP and capsaicin was prevented in guinea pig hearts by using colchicine. In this study, incubation with colchicine prolonged the stimulatory effects of CGRP and capsaicin in the guinea pig atrium. Colchicine acts probably by disruption of membrane bound proteins, thereby preventing the internalization (endocytosis) of CGRP receptors in the atria (Thyberg and Moskalewski, 1985; Vale, 1987). Therefore, inhibition of receptor endocytosis was a possible explanation for the lack of CGRP tachyphylaxis in the presence of colchicine. In our study, however, colchicine did not prevent the development of tachyphylaxis to CGRP. This discrepancy might be due to species and tissue differences. In addition, possible variation in coupling mechanisms for CGRP in atria and coronary arteries could be contributory to the discrepant findings. Another possible explanation is that colchicine may only affect the transport of ligand–receptor complex from membrane into the cytosol (endocytosis) without having a significant effect on the protein kinase-induced phosphorylation of G protein and/or β-arrestin-mediated uncoupling of G protein from the CGRP receptors in rat intramural coronary arteries. By using confocal immunofluorescence microscopy, recent studies have clearly demonstrated that CRL receptor was internalized (endocytosed) together with RAMPs via clathrin-coated vesicles following ligand exposure, and both the internalized molecules were targeted to the degradative pathway (Kuwasako et al., 2000; Hilairet et al., 2001). However, all these studies were performed on HEK cells with entirely different settings of intracellular components and effector proteins (e.g. ion channels) than those seen in smooth muscle cells of isolated vessels.

In order to mimic the reproducibility of CGRP-induced relaxation in U46619-precontracted coronary arteries, we used different vasodilator agents such as dbcAMP (a cell-permeable cAMP), forskolin and isoprenaline. In these experiments, however, there was no sign of tachyphylaxis to dbcAMP or forskolin during the second concentration–response curves with these compounds. In contrast to forskolin and dbcAMP, the coronary arteries developed tachyphylaxis to isoprenaline during the second concentration–response curve with isoprenaline. The desensitization pattern for isoprenaline (≈30% reduction in maximal response with no change in sensitivity) was similar to that with CGRP. All together, these results suggest that the development of tachyphylaxis to CGRP may occur at the G-protein level requiring receptor–agonist interaction.

It is well known that CGRP can cause vasodilation via a number of mechanisms (Sheykhzade and Nyborg, 2001). We have previously shown that CGRP exerts its direct vasodilator action (endothelium-independent) in rat intramural coronary arteries through activation of $CGRP_1$ receptors (Prieto et al., 1991; Sheykhzade and Nyborg, 1998a). By using radioimmunoassay and a selective inhibitor of cAMP-dependent PKA (Rp-cAMPS), we have shown that CGRP increases the intracellular cAMP level, thus leading to activation of cAMP–PKA pathway (data not shown). Furthermore, we have also demonstrated that CGRP activates large conductance calcium-activated potassium channels (BK_{Ca} channels) in rat intramural coronary arteries (Sheykhzade and Nyborg, 2001). It has been shown that the activity of BK_{Ca} channels is regulated by membrane potential and Ca^{2+} entry through L-type Ca^{2+} channels in coronary arterial myocytes (Guia et al., 1999). Since depolarization of the smooth muscle cell membrane with high concentration of extracellular K^+ eliminated the attenuation of CGRP-induced responses in rat intramural coronary arteries, this procedure could reactivate BK_{Ca} channels in the smooth muscle cell membrane. A recent study has shown that the activity of smooth muscle BK_{Ca} channels is regulated by protein kinases. In most cases, PKA and protein kinase G (PKG) stimulate the BK_{Ca} channel activity while PKC inhibits its activity (Schubert and Nelson, 2001).

The regulatory systems are even more complicated. A number of studies suggest that the K^+ channels can be activated by direct interaction between the G-protein subunit and the channel, without the involvement of second messengers or cAMP–PKA; either the free α-subunit or the βγ-subunit of the G protein may be the mediator, which controls the channel (Wickham and Clapham, 1995; see Beech, 1997 for review).

In our study, however, we cannot rule out the possibility that the attenuation of CGRP-induced relaxation is facilitat-

ed via PKC-mediated phosphorylation of either receptor or its associated ion channel (e.g. BK_{Ca} channel). Therefore, the development of tachyphylaxis to CGRP in rat intramural coronary arteries could be explained by either phosphorylation of the CGRP receptor or inactivation of BK_{Ca} channel. This is further supported by the observation that CGRP-induced tachyphylaxis was never observed in 36 mM K^+-depolarized coronary arteries and that the extent of CGRP-induced relaxation (efficacy) was significantly lower in 36 mM K^+-depolarized coronary arteries compared to U46619-precontracted coronary arteries. This is probably due to the fact that hyperpolarizing component of CGRP-induced relaxation is lost in high K^+-depolarized vascular smooth muscles. This phenomenon has also been observed in experiments with K^+ channel openers under the same experimental conditions (Beech, 1997).

In conclusion, the results of the present study demonstrate that CGRP-induced desensitization is a CGRP receptor-mediated event, which occurs at the G-protein level requiring receptor–agonist interaction. It seems that the CGRP-induced tachyphylaxis reaches a steady-state limit after the second exposure. Furthermore, CGRP-induced desensitization in rat intramural coronary arteries seems to be homologous in nature involving activation of CGRP receptor-related kinases than PKA. In addition, the desensitization pattern observed with CGRP is similar to that of isoprenaline. However, the type of kinases involved in CGRP-induced desensitization and the precise role of the membrane potential remains to be elucidated in the future.

Acknowledgements

This work is supported by Novo Nordisk Research Foundation.

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