Physiology and Pharmacology 26 (2022) 178-187
Original Article

RGS4 inhibition and the effects of adrenoceptor and cholinoceptor agonists on isolated left atrium and aorta of normal and diabetic rats

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ABSTRACT

Introduction: "Regulator of G protein signaling" (RGS) proteins are a family of various proteins that are expressed in different tissues and accelerate hydrolysis rate of GTP to GDP by several thousand-fold increase in GTPase activity of Gα subunit. Thus, they act as negative regulators of G protein-coupled receptors (GPCRs) signaling. In this study, the effect of CCG-50014, a RGS4 inhibitor, on isolated aorta and left atrium of normal and diabetic rats has been investigated.

Methods: Isolated aorta was treated with increasing concentrations of phenylephrine and acetylcholine. Isolated left atrium was treated with increasing concentrations of acetylcholine and isoprenaline; both in the absence and presence of CCG-50014. The pEC50 (negative logarithm of the concentration which produces half maximal response) and maximum response of each compound were extracted from concentration-response curves.

Results: Pre-treatment of aorta with CCG-50014 had no important effect on the response to phenylephrine and acetylcholine. CCG-50014 decreased isoprenaline inotropic potency on normal atrium but had no effect on its maximum response. In diabetic atrium, CCG-50014 dramatically reduced both the pEC50 and maximum response of isoprenaline. CCG-50014 did not affect normal atrium response to acetylcholine but in diabetic atrium, it caused a small yet significant decrease in the pEC50 of acetylcholine while increased its maximum relaxing effect.

Conclusion: It seems that RGS4 is not involved in the termination of GPCRs signaling in rat aorta. In atrium, RGS4 inhibition unexpectedly results in attenuation of β-adrenoceptor-mediated atrial contractility, which is much more prominent in diabetes.

Keywords:
Regulator of G-protein signaling (RGS) proteins
Atrium
Aorta
Diabetes
Isoprenaline

Introduction

G-protein coupled receptors (GPCRs) are one of the most important therapeutic targets for treatment of many diseases. Binding of a specific ligand to the GPCRs triggers a conformational change, which results in activation of the receptor, GDP replacement with GTP

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Received 10 October 2020; Revised from 28 March 2021; Accepted 26 July 2021

Citation: Goharinia M, Mirkhani H. RGS4 inhibition and the effects of adrenoceptor and cholinoceptor agonists on isolated left atrium and aorta of normal and diabetic rats. Physiology and Pharmacology 2022; 26: 178-187. http://dx.doi.org/10.52547/phypha.26.2.4

and dissociation of the Gβγ dimer from the Gα subunit. Both activated Gα (GTP-bound) and Gβγ detach from receptor and stimulate specific down-stream effector pathways. Termination of signal occurs by the intrinsic GTPase activity of Gα, which hydrolyzes GTP to GDP, results in Gα to return to its inactive (GDP-bound) state and reformation of the Gαβγ heterotrimer and re-association of the G-proteins with the GPCR (Hendriks-Balk et al., 2008).

The intrinsic rate of GTPase activity of Gα is relatively slow. In addition to this classical reaction, a family of GTPase activating proteins called "regulators of G-protein signaling (RGS) proteins" increase the intrinsic rate of GTP hydrolysis by several thousand-fold (Shaw et al., 2018; Siderovski and Kendall Harden, 2003). Thus, they act as negative regulators of GPCR signaling. The RGS proteins are a large family, which consists of more than 30 identified members in different tissues of mammals. Their expression is also different in health and disease states (Hendriks-Balk et al., 2008; Shaw et al., 2018). Theoretically, inhibition of RGS proteins could potentiate certain GPCR signaling and cellular response of natural or exogenous agonist ligands in a specific tissue or pathological states. It can also postulate that RGS inhibition could provide enhanced tissue specificity for the action of GPCR agonist by selectively increasing receptor response in tissues, which express a particular RGS protein.

RGS4 which is one of the first discovered RGS proteins plays an important role in regulation of cardiomyocyte (Gu et al., 2009) and smooth muscle (Cho et al., 2003; Wang et al., 2008) function. The 4-[(4-fluorophenyl) methyl]-2-(4-methylphenyl)-1, 2, 4-thiadiazolidine-3,5-dione (CCG-50014) is the most potent RGS inhibitor which has been identified so far (Blazer et al., 2011; Kimple et al., 2011; O'Brien et al., 2019; Senese et al., 2020; Shaw et al., 2018; Turner et al., 2012). Although several molecular and cellular studies on CCG-50014 have been performed (Monroy et al., 2013; O'Brien et al., 2019; Senese et al., 2020; Shaw et al., 2018; Vashisth et al., 2013), there are only few functional studies considering the role of RGS4 inhibition on the effect of agonists targeting the cardiovascular system (Chen et al., 2014). The studies in pathological conditions are very few and by searching the literature in our field of interest i.e. diabetes mellitus, revealed no similar results.

Cholinoceptors and adrenoceptors are GPCRs. At the vascular bed, the main subtype of cholinergic receptors is M3, which dominantly exists on endothelial cells and indirectly, by the release of nitric oxide, cause vasodilation. Although both alpha 1 and alpha 2 adrenoceptors exist on vascular smooth muscle cells, the dominant is of alpha 1 subtype that causes vasoconstriction. At cardiac tissue, M2 cholinoceptors are present and cause negative inotropism. Also, both beta 1 and beta 2 adrenoceptors are present that cause positive inotropism (Hendriks-Balk et al., 2008).

The present study has been carried out to investigate the effect of RGS4 inhibition on the action of cholinergic and adrenergic agonists on isolated rat aorta and left atrium. The results were compared with those of diabetic animals to clarify the role of diabetes on RGS4 function in cardiovascular system.

Materials and methods

Animals

Experiments were performed on Male *Sprague-Dawley* rats, weighing 180-220g, bred and kept by the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. The animals were kept in standard cages at controlled temperature (25°C) and fixed photoperiod 12h light/darkness condition. They had free access to food and tap water. The rats were randomly divided into normal and diabetic groups.

The animals were divided into normal and diabetic groups, 6-8 animals in each. To induce diabetes, animals were injected by a single intraperitoneal administration of streptozotocin (STZ) at the dose of 60mg/kg. Seven days after STZ injection, blood was drawn from the animal's tail using a needle and then blood glucose was measured using a glucometer (Accu-Chek Active). Rats with blood sugar greater than 300mg/dl were considered diabetics. All animal care and experimental procedures were approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethical approved ID: 2016285).

Drugs and chemicals

CCG-50014 was purchased from Calbiochem (San Diego, CA, USA). Acetylcholine, isoprenaline, phenylephrine and STZ were obtained from Sigma-Aldrich (UK). All the chemicals used for preparation of phys-

iological salt solution were of analytical grade. CCG-50014 was dissolved in dimethyl sulfoxide (DMSO). Final concentration of DMSO in organ bath was 0.1% DMSO that had no effect on tissue responses in preliminary studies. All other drugs were dissolved in distilled water.

Tissue studies

After eight weeks of diabetes induction, the blood sugar was measured once again and animals were deeply anaesthetized using high dose of ketamine/xylazine (80/10 mg/kg, IP). The thorax was opened by means of a midline incision and the heart and descending thoracic aorta were swiftly dissected. After removal of superficial fat and surrounding connective tissue, we made sure not to damage the endothelium. Then the aorta was divided into two rings of about 2-3 mm in length. Each aortic ring was mounted between two parallel wires in organ bath. Left atria were dissected free from the hearts and mounted between two parallel stainless steel electrodes in organ bath. The left atria were paced by the following stimulation specifications: frequency= 1Hz, Pulse width= 5ms and voltage= 20% above threshold.

The organ bath contained 20ml of physiological salt solution with the following composition (mM): NaCl 119, KCl 4, KH_2PO_4 1.2, $CaCl_2$ 3.3, $MgSO_4$ 2.4, $NaHCO_3$ 25 and D-glucose 11. The solution temperature was maintained at 37°C and bubbled continuously with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. The resting tension of tissues was adjusted to 0.5g for an equilibration period of 60min after which the main experiment was carried out. The tension of the preparations was recorded via an isometric force transducer (K 30, Hugo Sachs Elektronik, Germany) on a PC software (HSE-ACAD, Hugo Sachs Electronik, Germany). Bathing solution was exchanged every 10min.

Isolated aorta

To investigate the effect of CCG-50014 on vascular α1-adrenergic receptor stimulation, at first the aortic rings were exposed to cumulative concentrations of phenylephrine. After washing out and reaching the baseline tension, aortic rings were pre-incubated with CCG-50014 (30nM) for 10min (O'Brien et al., 2019). Thereafter, the tissues were treated again with cumulative concentrations of phenylephrine. In both conditions, the concentration-response curves were plotted. Then the pEC50 values (negative logarithm of the concentration that produced 50% of maximum response) and maximum tension that was produced were calculated.

In another set of experiments, the vasorelaxant effect of acetylcholine (as a vascular muscarinic-receptor agonist) was studied. In these experiments, aortic rings were pre-contracted with sub-maximal concentration (concentration that produced 60–70% of maximal response) of phenylephrine. Thereafter, the tissues were treated with cumulative concentrations of acetylcholine and the pEC50 and maximum relaxant response were calculated. After washing out and reaching the baseline tension the tissues were pre-incubated for 10min period with CCG-50014. Subsequently, the same protocol was applied once again.

Isolated left atrium

To examine the effect of CCG-50014 on the atrial β-adrenergic and muscarinic receptors stimulation, at first the isolated left atrium was exposed to cumulative concentrations of isoprenaline and increasing atrial contractions were recorded. After washing out and maintaining the baseline contractions, the left atrium was pre-incubated with CCG-50014 (30nM) for 10min. Thereafter, the tissues were treated once again with cumulative concentrations of isoprenaline. In both conditions, the concentration-response curves were plotted. Then the pEC50 values (negative logarithm of the concentration that produced 50% of maximum contraction) and maximum contraction that was produced were calculated.

In another set of experiments, the negative inotropic effect of acetylcholine was studied. The protocol was similar to isoprenaline experiment, except that the acetylcholine reduced atrial contractility. Therefore, its pEC50 (negative logarithm of the concentration that produced 50% of maximum relaxation) and maximum inhibitory effect were recorded and calculated instead.

Statistical analysis

All the values were expressed as the mean±SEM. The pEC50s were calculated based on the best-fit method using Curve Expert software (Version.1.3). Statistical analysis was done using SPSS software (Version. 16). Results obtained from normal and diabetic tissues were compared using independent-samples T test and Mann-Whitney test, when appropriate. The effect of

CCG-50014 on response of each preparation was compared to the same preparation response in the absence of this agent, using paired sample T-test. Differences were considered to be statistically significant when *P*-value was <0.05.

Results

Effect of diabetes on isolated aorta and left atrium

After eight weeks, the STZ-treated rats had a mean serum glucose level of 430±24 mg/dl, while glucose levels in the control group were 122±11 mg/dl. Diabetes resulted in a significant reduction in potency of phenylephrine on aortic contractions (*P*=0.030), whereas, it had no effect on its maximum vasomotion effect (Table 1). Diabetes caused a significant reduction in potency (*P*=0.046) and maximum relaxing response (*P*=0.046) of acetylcholine, in aortic rings (Table 1).

On atrial contractions, diabetes had no significant effect on the potency of isoprenaline (Table 2). Diabetes reduced the maximum inotropic effect of isoprenaline

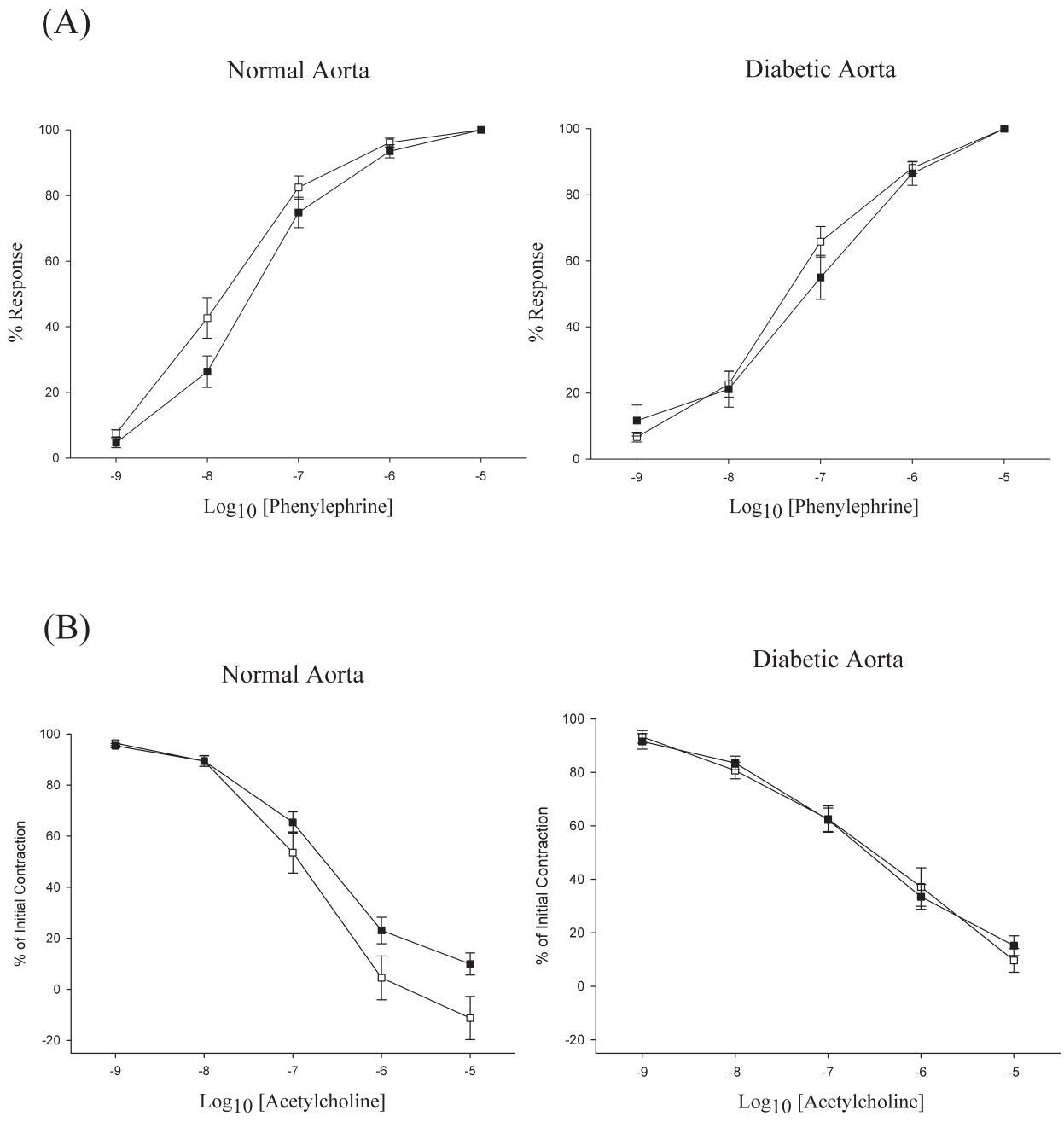

FIGURE 1. Effect of RGS4 inhibition on the response of isolated aorta to phenylephrine and acetylcholine. The responses of isolated aorta to phenylephrine (A) and acetylcholine (B) in the media with (filled square) and without (open square) CCG-50014 have been shown. Each point represents the mean±SEM (n= 6-8). For statistical comparisons, see Table 1.

TABLE 1: CCG-50014 effects on the potencies and maximum effects of phenylephrine and acetylcholine in isolated aorta. Data are presented as the mean ± S.E.M. (n= 6-8).

Agent	Parameter	Groups	Control	CCG-50014 treatment	*P*-value (before vs. after treatment)
Phenylephrine	pEC_{50} (M)	Normal	7.88 ± 0.13	7.53 ± 0.11	0.059
		Diabetic	7.41 ± 0.13	7.16 ± 0.20	0.311
		P-value (normal vs. diabetics)	0.030*		
	Emax (mg)	Normal	936.83 ± 91.53	898.50 ± 115.41	0.800
		Diabetic	837.83 ± 184.96	752.33 ± 164.80	0.737
		P-value (normal vs. diabetics)	0.642		
Acetylcholine	pEC_{50} (M)	Normal	6.87 ± 0.13	6.57 ± 0.10	0.101
		Diabetic	6.36 ± 0.19*	6.53 ± 0.14	0.498
		P-value (normal vs. diabetics)	0.046		
	(%) Emax	Normal	111.25 ± 8.46	90.06 ± 4.31*	0.043
		Diabetic	90.31 ± 4.43*	84.81 ± 3.70	0.357
		P-value (normal vs. diabetics)	0.046		

*Significant difference ($P< 0.05$).

by about 30% but the difference did not reach statistical significance ($P= 0.075$, Table 2). Diabetes caused a small but statistically significant reduction in potency of acetylcholine on atrial contractions ($P=0.048$, Table 2). Meanwhile, it had no significant effect on the maximum relaxant effect of acetylcholine (Table 2).

Effect of CCG-50014 on phenylephrine and acetylcholine responses in isolated aorta

Pre-treatment of tissues with CCG-50014 had no significant effect on the potency of phenylephrine and acetylcholine in normal and diabetic aorta. The same results were achieved for maximum vasomotion effects except that in normal group, CCG-50014 had significantly ($P=0.043$) reduced acetylcholine maximum relaxing effect (Figure 1 and Table 1).

Effect of CCG-50014 on isoprenaline response in left atrium

Pre-treatment of normal atrium with CCG-50014 attenuated isoprenaline potency significantly ($P=0.025$), but had no effect on maximum response. In diabetic atrium, CCG-50014 treatment reduced isoprenaline inotropic potency so dramatically, that this value could not be calculated. It also significantly ($P=0.014$) reduced maximum effect of isoprenaline (Fig. 2A and Table 2).

Effect of CCG-50014 on acetylcholine response in left atrium

Pre-treatment of normal atrium with CCG-50014 had no effect on potency and maximum response of acetylcholine. In diabetic atrium, pre-treatment of tissues with CCG-50014 significantly reduced the potency of acetylcholine ($P=0.036$) but increased its maximum relaxing effect (Figure 2 and Table 2).

Discussion

RGS proteins accelerate the rate of GTP to GDP hydrolysis reaction, which is mediated by the Gα subunit. By this mean, they reduce the lifespan of GPCR signaling pathway. The present study has been carried out to investigate the effect of CCG-50014, a specific RGS4 inhibitor, on the adrenergic- and cholinergic-mediated responses of isolated rat atrium and aorta. Phenylephrine contracted isolated rat aorta. However, its potency to contract diabetic vessels was significantly lesser than

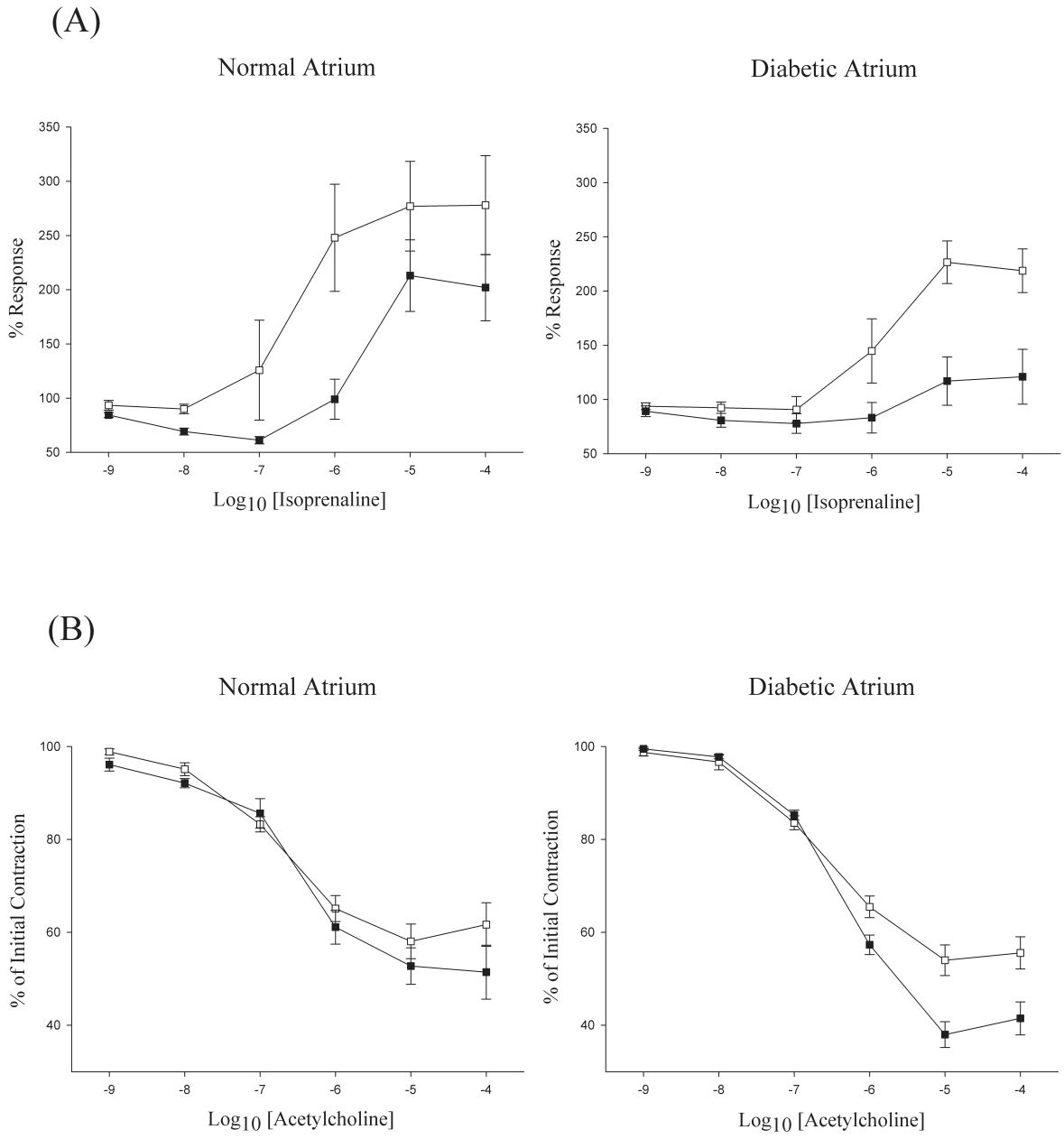

FIGURE 2. Effect of RGS4 inhibition on the response of isolated atrium to isoprenaline and acetylcholine. The responses of isolated left atria to isoprenaline (A) and acetylcholine (B) in the media with (filled square) and without (open square) CCG-50014 have been shown. Each point represents the mean±SEM (n= 6-8). For statistical comparisons, see Table 2.

its effect on normal ones. Other studies are contradictory (Hink et al., 2001; Zeydanli et al., 2011). Pre-incubation of tissues with CCG-50014 had no significant effect on the aortic response to phenylephrine.

Phenylephrine binds to the α1-adrenoceptors on vascular smooth muscle cells and stimulates Gq proteins. The liberated Gαq via stimulation of phospholipase C, increases cytosolic level of inositol-1,4,5-trisphosphate and diacylglycerol. Finally, it results in increase of intracellular calcium level that contracts the vessel (Weber and Macleod, 1997). The obtained results indicate that RGS4 has no role in the termination of Gαq signaling in vascular bed. Nevertheless, it must be mentioned that the RGS4 expression in thoracic rat aorta has been previously reported. However, its expression level has been at least 12 times lower than that of RGS5 (Wang et al., 2008).

As expected, the potency and maximal relaxing effect of acetylcholine was lesser in diabetic isolated rat aorta. Acetylcholine binds to M3-cholinoceptors of vascular endothelial cells and by stimulating Gq, increases endothelial nitric oxide synthase (eNOS) activity and nitric

TABLE 2: CCG-50014 effects on the potencies and maximum effects of isoprenaline and acetylcholine in isolated left atria. Data are presented as the mean ± S.E.M. (n= 6-8).

Agent	Parameter	Groups	Control	CCG-50014 treatment	*P*-value (before vs. after treatment)
Isoprenaline	pEC_{50} (M)	Normal	6.67 ± 0.16	5.95 ± 0.18*	0.025
		Diabetic	6.33± 0.13	Not calculated	-
		P-value (normal vs. diabetics)	0.145		
	Emax (%)	Normal	307 ± 48	237 ± 29	0.258
		Diabetic	211 ± 22	117 ± 22*	0.014
		P-value (normal vs. diabetics)	0.075		
Acetylcholine	pEC_{50} (M)	Normal	6.72 ± 0.03	6.67 ± 0.04	0. 346
		Diabetic	6.57 ± 0.07*	6.38 ± 0.03*	0.036
		P-value (normal vs. diabetics)	0.048		
	Emax (%)	Normal	40 ± 4	45 ± 4	0.378
		Diabetic	44 ± 2	62 ± 3*	0.000
		P-value (normal vs. diabetics)	0.477		

*Significant difference ($P< 0.05$).

oxide (NO) production. NO mediates endothelium-dependent vascular relaxation (Ajay et al., 2006; Machha et al., 2007). In diabetes, diminution of endothelium-dependent relaxation seems to be as a result of decreased number of muscarinic receptors and eNOS expression (Kazuyama et al., 2009). Vascular tissues that were pre-incubated with CCG-50014, showed no significant changes in response to acetylcholine. It can be determined that in addition to vascular smooth muscle cells, the RGS4 seems to have no effect on the termination of Gαq action in vascular endothelial cells as well. On the other hand, it is worth mentioning that inhibition of RGS4 reduced maximum effect of acetylcholine in normal aortic tissues, an observation that we have no explanation for.

Isoprenaline increased contractility of both normal and diabetic isolated rat left atria. While its potency did not differ between normal and diabetic tissues, its maximal inotropic effect was about 50% less than diabetic atrium. Nevertheless, this difference did not reach a statistical significance. Isoprenaline binds to β-adrenoceptors, stimulates Gs protein and increase adenylate cyclase activity. It finally increases intracellular cAMP level and shows positive chrono- and inotropic effects (Altan et al., 2007; Thackeray et al., 2012). If RGS4 deactivated Gαs, the CCG-50014 must inhibit this reaction and increase isoprenaline inotropic action. Also, if RGS4 had no role on Gαs action, its inhibition would have no effect on the isoprenaline action. Unexpectedly, in practice, pre-incubation of atrial tissues with CCG-50014 significantly reduced inotropic action of isoprenaline. In diabetic tissues, this inhibitory effect was so prominent that isoprenaline practically showed no inotropic effect and we were unable to determine its potency.

Most likely, this unexpected observation was due to alteration in the proportion of the β-adrenoceptor population in the myocardium. The β1-adrenoceptors are the main adrenoceptors in heart. Nevertheless, there are some β2-and β3-adrenoceptors on the myocardial cells, which are stimulated by isoprenaline too. It is reported that these receptors are bound to both Gs and Gi proteins. It can be assumed that inhibition of RGS4 potentiates inhibitory Gi-mediated arm while it has no action on stimulatory Gs-mediated action of isoprenaline. It is worth mentioning that several studies have reported that, RGS4 proteins have no effect on the termination of Gαs action (Hao et al., 2006; Owen et al., 2001; Tesmer et al., 1997; Wang et al., 2008). Interestingly, it was report-

ed that the population of β2- and β3-adrenoceptors are increased in diabetes (Altan et al., 2007; Thackeray et al., 2012), a finding that can explain our observation in diabetic tissues, i.e. in diabetes and in the presence of a RGS4 inhibitor, the potentiation of the inhibitory arm of β-adrenergic stimulation (via β2 and β3-adrenoceptors) is greater than the effects observed in normal tissues.

Our experimental design cannot rule out other possible mechanism of actions of CCG-50014 (e.g. β-adrenergic blocking effects). However, it is worth mentioning that in an earlier study with another RGS4 inhibitor (CCG-4986), similar results were obtained. So, it seems that the observed results are due to the inhibition of RGS4. Due to inadequate amount of CCG-4986, the study was carried out merely on two normal and four diabetic isolated atria. Hence, we decided not to publish the results, abandoned the study and switched to another RGS4 inhibitor, i.e. CCG-50014. In the present study, the potency of acetylcholine to decrease the contractility of isolated diabetic atrium was lower than the obtained results on normal tissues. This difference was minute but statistically significant. This effect may be due to reduced number and sensitivity of cardiac muscarinic receptors in diabetes (Carrier et al., 1984; Kofo-Abayomi and Lucas, 1987). However, contradictory results were also published (Dall'ago et al., 2007; Wald et al., 1998). The acetylcholine action on the heart is mediated by muscarinic M2 receptors. These receptors are coupled to Gi and their stimulation results in Gαi release, inhibition of adenylate cyclase action, decrease of cAMP level, and finally negative inotropism. Also, the liberated Gβγ subunit can directly open inwardly-rectifying potassium channels and reduce atrial contractility (Krejčí et al., 2004).

Pre-incubation of normal atrium with CCG-50014 had no effect on the negative inotropic action of acetylcholine. Such intervention in diabetic tissues, resulted in a little but statistically significant decrease in potency of acetylcholine (lower negative inotropism), while increased its maximum action (higher maximum negative inotropism). These results may imply a greater role of other pathways rather than Gαi release, on the action of acetylcholine in atrial tissue (i.e. Gβγ action on potassium channels, especially in normal tissues). Also, there are different subtypes of Gi proteins in cardiac tissue (Mittmann, 2003). It can be postulated that these subtypes are modulated differently by various agonists and different RGS proteins. Clearly, further investigation is required in this regard. Obtained results revealed that inotropic effect of sympathomimetic agents might be altered in diabetic patients. In addition, modulation of the cardiac-adrenergic signaling pathways may produce different effect in diabetes in comparison to normal condition.

Conclusion

It can be concluded that RGS4 has no significant role on the modulation of vascular action of G-proteins that are coupled to the adrenergic and cholinergic receptors. In contrast, RGS4 exists in rat cardiac tissue and its inhibition unexpectedly decreases β-adrenoceptor mediated atrial contractility, an effect that is much greater in diabetic tissues. Inhibition of RGS4 has no significant effect on the cholinoceptor mediated action on the atrial tissue except only a minor effect was observed in diabetic tissue.

Acknowledgments

The present work was supported by the Research Grant from the Shiraz University of Medical Sciences, Iran (Grant number: 3110). The authors would like to thank Azar Purkhosrow and Azam Bagheri for their technical support. The authors would also like to thank the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for their valuable assistant in editing this article.

Conflict of interest

The authors declare that none of them has any conflicts of interest with the contents of this article.

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