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SPECIAL REPORT

Adenosine A_1 receptor stimulation inhibits α_1-adrenergic activation of the cardiac sarcolemmal Na^+/H^+ exchanger

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Sarcolemmal Na^+/H^+ exchanger (NHE) activity is increased by stimulation of G_q protein-coupled receptors (G_qPCRs), but the roles of other GPCRs are largely unknown. We determined the effects of N-[(1S,trans)-2-hydroxycyclopentyl]adenosine (GR79236), a selective agonist of the G_iPCR adenosine A_1 receptor, on sarcolemmal NHE activity in adult rat ventricular myocytes ($n=8-10$ per group). NHE activity was indexed by the H^+ efflux rate after intracellular acidification, measured by microepifluorescence. GR79236 alone (0.01–10 μM) had no effect on NHE activity. However, co-administration of GR79236 inhibited, in a concentration-dependent manner, the stimulation of NHE activity by the α_1-adrenoceptor agonist phenylephrine (10 μM). The inhibitory effect of GR79236 (10 μM) was abolished by (1) the selective A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine (0.1 μM), confirming an A_1 receptor-mediated action, and (2) pre-treatment with pertussis toxin (5 μg ml^{-1} for 60 min), indicating a G_i protein-mediated mechanism. Our data suggest the existence of inhibitory crosstalk between the G_iPCR adenosine A_1 receptor and the G_qPCR α_1-adrenoceptor in the regulation of sarcolemmal NHE activity.
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Abbreviations: AR, adrenoceptor; CABG, coronary artery bypass graft; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GPCR, G protein-coupled receptor; NHE, Na^+/H^+ exchanger; PAR1, protease-activiated receptor 1

Introduction The sarcolemmal Na^+/H^+ exchanger (NHE) is the ubiquitously expressed product of the NHE1 gene (Fliegel & Dyck, 1995) and is an important H^+ extrusion mechanism that contributes to the control of intracellular pH (pH_i) in cardiac myocytes (Leem *et al*., 1999). Nevertheless, increased activity of the sarcolemmal NHE has been implicated in the development of myocardial injury and dysfunction during ischaemia and reperfusion, and NHE1-selective pharmacological inhibitors have been shown to be cardioprotective in this setting in numerous animal studies (Avkiran, 1999a). Recent clinical data suggest that one such inhibitor, cariporide, may provide cardioprotective benefit in patients with anterior myocardial infarction who receive early reperfusion by direct coronary angioplasty (Rupprecht *et al*., 2000) and in high-risk patients who undergo global myocardial ischaemia and reperfusion during coronary artery bypass graft (CABG) surgery (Théroux *et al*., 2000).

Sarcolemmal NHE activity is regulated primarily by pH_i and increases markedly in response to intracellular acidosis (Leem *et al*., 1999); it is also subject to stimulation by agents that act *via* G_q protein-coupled receptors (G_qPCRs), such as α_1-adrenoceptor (α_1-AR) agonists, endothelin, thrombin and angiotensin II (reviewed by Avkiran, 1999b). However, there is little known about the regulation of sarcolemmal NHE activity by receptors outside the G_qPCR family.

Adenosine is an adenine nucleoside that has been shown to possess cardioprotective efficacy in animal models of myocardial ischaemia and reperfusion (Lasley & Mentzer, 1996). The therapeutic potential of adenosine has also recently been tested in humans, as an adjunct to thrombolysis in patients with acute myocardial infarction (Mahaffey *et al*., 1999) and as an additive to cardioplegia in patients undergoing CABG surgery (Mentzer *et al*., 1999), with encouraging results. Although adenosine can potentially afford myocardial protection during ischaemia and reperfusion through the stimulation of multiple adenosine receptors in a variety of cell types, direct stimulation of myocardial A_1 receptors appears to be an important component of such protection (Lasley & Mentzer, 1996). Indeed, hearts from transgenic mice with cardiac-specific overexpression of the A_1 receptor have been shown to exhibit reduced susceptibility to ischaemia and reperfusion-induced injury (Matherne *et al*., 1997). The mechanisms through which the myocardial A_1 receptor, which is a member of the G_iPCR family, exerts protection are unclear.

Our recent work has shown that stimulation of another G_iPCR, the angiotensin AT_2 receptor, inhibits sarcolemmal NHE activation *via* the G_qPCR angiotensin AT_1 receptor (Gunasegaram *et al*., 1999). The possibility exists that adenosine A_1 receptors may also initiate signalling events that negatively regulate sarcolemmal NHE activity. Therefore, the present study was undertaken to determine the effects of adenosine A_1 receptor stimulation by the selective agonist N-[(1S,trans)-2-hydroxycyclopentyl]adenosine (GR79236) (Gurden *et al*., 1993) on sarcolemmal NHE activity, in the resting state and following G_qPCR stimulation. Preliminary results of the study have been published in abstract form (Yokoyama & Avkiran, 2000).

Methods This investigation was performed in accordance with the Home Office 'Guidance on the Operation of the Animals (Scientific Procedures) Act 1986', published by HMSO, London.

Isolation of ventricular myocytes Ventricular myocytes were isolated from hearts of adult (200–250 g body weight) male Wistar rats (B&K Universal, Hull, U.K.) by enzymatic

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digestion, as previously described (Yasutake *et al*., 1996; Yokoyama *et al*., 1998; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000).

Determination of sarcolemmal NHE activity Sarcolemmal NHE activity was determined in single myocytes loaded with the pH-sensitive fluoroprobe cSNARF-1, using an established microepifluorescence technique (Yasutake *et al*., 1996; Yokoyama *et al*., 1998; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000). Cells were superfused with bicarbonate-free Tyrode's solution (34°C) throughout each experiment and the rate of acid efflux (J_H) was used as the index of sarcolemmal NHE activity. J_H was determined at an intracellular pH (pH_i) of 6.90 ($J_{H6.9}$), during recovery from intracellular acidosis that was induced by transient exposure to NH_4Cl (see below).

Experimental protocols Myocytes were subjected to intracellular acidosis by a 3-min exposure to 20 mmol/l NH_4Cl (first acid pulse), which was repeated 15 min later (second acid pulse) (Yasutake *et al*., 1996; Yokoyama *et al*., 1998; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000). In control cells, both acid pulses occurred in the absence of any drug. When studying the effects of GR79236 (gift from GlaxoWellcome, Stevenage, U.K.) alone, this was present during the second acid pulse. When studying the effects of GR79236 on the response to phenylephrine or thrombin (both from Sigma, Poole, U.K.), the G_qPCR agonist was given during the second acid pulse and GR79236 was present from 6 min before this pulse. 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX; Sigma), when used, was given concomitantly with GR79236. Stock solutions of drugs, except DPCPX, were dissolved in deionized water and were diluted ($\geqslant$1000 fold) in Tyrode's solution to obtain appropriate final concentrations; the DPCPX stock solution was dissolved in dimethylsulphoxide (final concentration 0.005%). Phenylephrine solutions contained 1 μM atenolol (Sigma), to preclude β_1-AR-mediated actions (Yokoyama *et al*., 1998). When required, cells were pre-treated with 5 μg ml^{-1} pertussis toxin (Sigma) for 60 min, in order to inactivate G_i proteins (Obayashi *et al*., 1997).

Statistical analysis Data are expressed as mean$\pm$s.e.mean. Each protocol comprised 4–8 study groups and experiments within it were carried out in a randomized manner (n=8–10 myocytes per group, obtained from 5–15 hearts). For intergroup comparison of the changes in $J_{H6.9}$ ($\Delta J_{H6.9}$) in response to vehicle or drug(s), data were subjected to ANOVA; if a significant difference was found, further analysis was by Dunnett's test, to compare each treatment group with the control group. $P<0.05$ was considered significant.

Results *Effects of GR79236 alone* $J_{H6.9}$ values during recovery from the first acid pulse in control cells and in those that received 0.01, 0.1, 1 or 10 μM GR79236 (n=10 cells per group, from 10 hearts) were 3.56$\pm$0.31, 3.28$\pm$0.45, 3.71$\pm$0.70, 4.36$\pm$0.77 and 3.06$\pm$0.41 mM min^{-1}, respectively (NS). There was no significant change in $J_{H6.9}$ during the second acid pulse in any group, with $\Delta J_{H6.9}$ values of 13$\pm$19, 18$\pm$25, 13$\pm$20, 4$\pm$12 and 10$\pm$31%, respectively (NS). These findings indicate that acute exposure to GR79236 does not significantly affect sarcolemmal NHE activity in the resting state.

Effects of GR79236 on the response to phenylephrine We next determined whether exposure to GR79236 affects the stimulation of sarcolemmal NHE activity by phenylephrine. $J_{H6.9}$ values during the first acid pulse did not differ significantly between the eight study groups (n=10 cells per group, from 15 hearts) and were as follows: 3.18$\pm$0.48 mM min^{-1} in control cells; 3.21$\pm$0.44 mM min^{-1} in cells that received phenylephrine (10 μM) alone; 2.5$\pm$0.29, 2.78$\pm$0.50, 2.73$\pm$0.69 and 3.33$\pm$0.27 mM min^{-1}, respectively, in cells that received phenylephrine (10 μM) in combination with 0.01, 0.1, or 10 μM GR79236; 3.80$\pm$0.69 and 3.13$\pm$0.49 mM min^{-1}, respectively, in cells that received 1 or 10 μM GR79236 alone. Figure 1 illustrates the $\Delta J_{H6.9}$ values in these groups. Consistent with our earlier findings (Yokoyama *et al*., 1998; Snabaitis *et al*., 2000), phenylephrine alone produced a large and significant increase in sarcolemmal NHE activity. GR79236 inhibited the NHE-stimulatory effect of phenylephrine in a concentration-dependent manner, such that the α_1-AR agonist no longer produced a significant increase in NHE activity when given in combination with 1 or 10 μM GR79236. Once again, 1 or 10 μM GR79236 alone failed to produce a significant change in sarcolemmal NHE activity. These data indicate that exposure to GR79236 inhibits α_1-AR-mediated stimulation of sarcolemmal NHE activity.

Reversal of the inhibitory effect of GR79236 by DPCPX In order to confirm that the inhibitory effect of GR79236 on the α_1-adrenergic response was mediated *via* the A_1 receptor, rather than through a non-specific action, we tested the reversibility of this effect by the selective A_1 receptor antagonist DPCPX. $J_{H6.9}$ values during the first acid pulse did not differ significantly between the five study groups (n=9 cells per group, from seven hearts) and ranged between 2.48$\pm$0.44 and 3.08$\pm$0.30 mM min^{-1}. Figure 2 illustrates the $\Delta J_{H6.9}$ values. As expected, 10 μM phenylephrine produced a significant increase in NHE activity and this response was again abolished when phenylephrine was given in combination with 10 μM GR79236. However, when GR79236 was given concomitantly with 0.1 μM DPCPX, the A_1 agonist was no longer able to inhibit α_1-AR-mediated stimulation of sarcolemmal NHE activity. DPCPX alone was without effect. These findings confirm that the inhibitory effect of GR79236 on α_1-adrenergic stimulation of sarcolemmal NHE activity was mediated *via* the A_1 receptor.

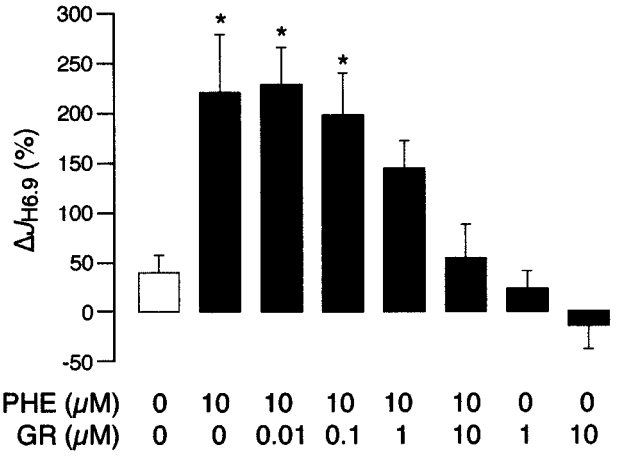

Figure 1 The change in H+ efflux rate at pH_i 6.90 ($\Delta J_{H6.9}$) in the control group and in response to phenylephrine (PHE) and GR79236 (GR), alone or in combination. The protocol comprised 80 myocytes (n=10 per group) obtained from 15 hearts. *$P<0.05$ vs control.

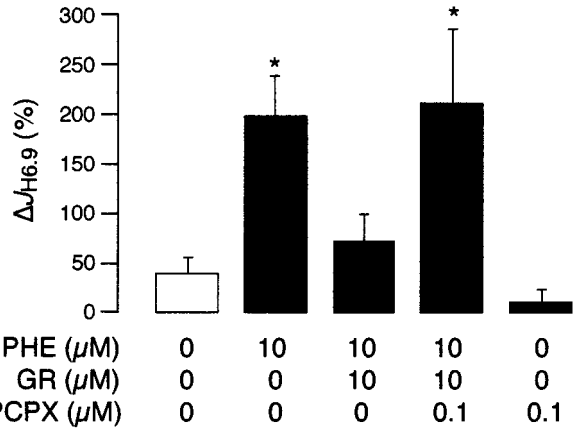

Figure 2 The change in H+ efflux rate at pH_i 6.90 ($\Delta J_{H6.9}$) in the control group and in response to phenylephrine (PHE), GR79236 (GR) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), alone or in combination. The protocol comprised 45 myocytes ($n=9$ per group) obtained from seven hearts. *$P<0.05$ vs control.

Reversal of the inhibitory effect of GR79236 by pertussis toxin In order to probe the signalling mechanisms distal to the A_1 receptor that mediate the inhibitory effect of GR79236, we examined the consequences of inactivating the G_i protein, by a 60-min pertussis toxin pre-treatment. $J_{H6.9}$ values during the first acid pulse were similar between the eight study groups ($n=8$ cells per group, from 14 hearts) within this protocol and ranged between 2.81 ± 0.47 and 3.30 ± 0.40 mM min^{-1}. Figure 3 illustrates the $\Delta J_{H6.9}$ values. Our observations in cells pre-treated with vehicle for 60 min were essentially identical to those illustrated in Figure 1; 10 μM phenylephrine significantly increased sarcolemmal NHE activity and this response was abolished by 10 μM GR79236 (Figure 3, top panel). In contrast, in cells pre-treated with pertussis toxin for 60 min, 10 μM GR79236 was no longer able to inhibit the stimulation of sarcolemmal NHE activity by 10 μM phenylephrine (Figure 3, bottom panel). It appears therefore that G_i protein activation is a critical step in the signalling mechanisms downstream of the A_1 receptor that mediate the inhibitory effect of GR79236 on α_1-adrenergic stimulation of sarcolemmal NHE activity.

Effects of GR79236 on the response to thrombin Finally, we determined whether GR79236 could inhibit the stimulation of sarcolemmal NHE activity by another G_qPCR agonist, namely thrombin (Yasutake *et al*., 1996). In this protocol also, $J_{H6.9}$ values during the first acid pulse were similar between the four study groups (range from 3.75 ± 0.75 to 4.13 ± 0.71 mM min^{-1}; $n=8$ cells per group, from five hearts). As expected from our earlier work (Yasutake *et al*., 1996), relative to control ($\Delta J_{H6.9}$ $14\pm19\%$), 5 u ml^{-1} thrombin significantly increased sarcolemmal NHE activity ($\Delta J_{H6.9}$ $126\pm34\%$); however, this response was attenuated by 1 μM GR79236 ($\Delta J_{H6.9}$ $50\pm22\%$) and abolished by 10 μM GR79236 ($\Delta J_{H6.9}$ $26\pm16\%$). These data suggest that GR79236 can inhibit the stimulation of sarcolemmal NHE activity by multiple G_qPCR agonists.

Discussion Although the anti-β_1-adrenergic effect of adenosine A_1 receptor stimulation (thought to be mediated primarily through a G_i protein-mediated reduction in adenylyl cyclase activity) is well established, the present data represent the first demonstration of an anti-α_1-adrenergic effect of A_1 receptor stimulation in cardiac myocytes. Specifically, our data have

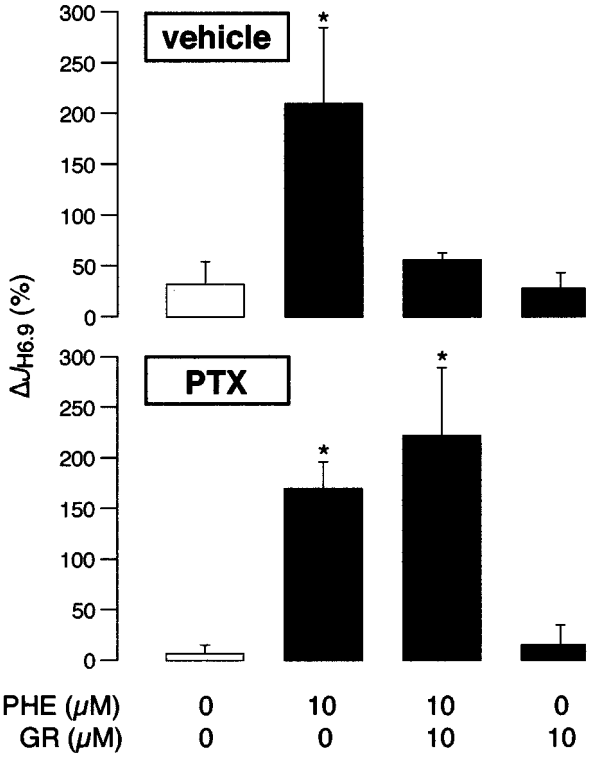

Figure 3 The change in H+ efflux rate at pH_i 6.90 ($\Delta J_{H6.9}$) in the control group and in response to phenylephrine (PHE) and GR79236 (GR), alone or in combination. The protocol comprised 64 myocytes ($n=8$ per group) obtained from 14 hearts. The cells were pre-treated for 60 min with either vehicle (top panel) or 5 μg ml^{-1} pertussis toxin (PTX; bottom panel). *$P<0.05$ vs control.

shown that the A_1 agonist GR79236 inhibits α_1-adrenergic stimulation of sarcolemmal NHE activity, a response that is mediated *via* the α_{1A}-AR (Yokoyama *et al*., 1998). The inhibitory effect of GR79236 occurs *via* stimulation of the A_1 receptor (based on its reversibility by the A_1 antagonist DPCPX) and requires a functional G_i protein (based on its abolition following pertussis toxin pre-treatment). Importantly, the effect was not limited to inhibition of the α_1-adrenergic response, since GR79236 also inhibited the stimulation of sarcolemmal NHE activity by thrombin, a response that is mediated by the thrombin receptor (Yasutake *et al*., 1996), now termed protease-activated receptor 1 (PAR1). Since both the α_{1A}-AR and PAR1 are members of the G_qPCR family, our data suggest the existence of a novel inhibitory crosstalk mechanism between the G_iPCR adenosine A_1 receptor and multiple G_qPCRs, at least in rat ventricular myocytes. If confirmed, such a mechanism may have implications beyond the regulation of sarcolemmal NHE activity.

In view of the important role that sarcolemmal NHE activity is believed to play in the development of myocardial injury and dysfunction during ischaemia and reperfusion (Avkiran, 1999a), inhibition of G_qPCR-mediated NHE activation is likely to contribute to the mechanisms underlying the cardioprotective effects of adenosine A_1 receptor stimulation, by GR79236 (Louttit *et al*., 1999) and other interventions (Lasley & Mentzer, 1996). However, during ischaemia and reperfusion, sarcolemmal NHE activity may be stimulated not only by a variety of endogenous mediators (e.g. catecholamines, thrombin, endothelin) that act *via* G_qPCRs, but also by factors such as oxidant stress and exposure to lipid metabolites (Avkiran, 1999b). Therefore, it would be of interest to

determine whether A_1 receptor stimulation attenuates the stimulation of sarcolemmal NHE activity by these additional factors.

Of relevance to the present work, increased sarcolemmal NHE activity and consequent increases in pH_i and/or intracellular [Na^+] have been suggested also to be causally involved in the positive inotropic and hypertrophic consequences of myocardial α_1-AR stimulation. In the light of our data, it is reasonable to expect that adenosine A_1 receptor stimulation may attenuate these effects. Indeed, there is preliminary evidence that, in rat ventricular myocytes stimulated at 0.5 Hz, GR79236 inhibits the positive effects of phenylephrine on (1) cell shortening under normal conditions, and (2) the recovery of cell shortening following intracellular acidosis (P. Krishnan and J.C. Kentish, King's College London, personal communication).

Our data suggest that the inhibitory effect of adenosine A_1 receptor stimulation on the α_1-adrenergic response is mediated *via* the G_i protein. However, the relevant mechanisms that are downstream of G_i protein activation are unclear. In view of the inability of GR79236 to significantly reduce NHE activity when given alone (e.g. Figures 1 and 3), the inhibitory effect is unlikely to reflect functional antagonism through an independent signalling pathway. Rather, it appears that A_1 receptor stimulation may initiate events that interfere with the NHE-regulatory signalling mechanisms that lie distal to the α_{1A}-AR. Our recent work has shown that α_{1A}-AR-mediated stimulation of sarcolemmal NHE activity in rat ventricular myocytes requires the activation of both protein kinase C (PKC) and extracellular signal-regulated kinases 1 and 2 (Snabaitis *et al*., 2000). PKC activation has been shown to be necessary also for thrombin-induced stimulation of sarcolemmal NHE activity (Yasutake *et al*., 1996). In this context, it is interesting to note that stimulation of adenosine A_1 receptors in rat isolated hearts and ventricular myocytes attenuates the effects of dioctanoylglycerol, a direct activator of PKC, on contractility and Ca^{2+} handling (Narayan *et al*., 1998). Furthermore, there is recent evidence that, in rat ventricular myocytes, activation of protein phosphatases contributes to the anti-β_1-adrenergic mechanisms of A_1 receptor stimulation (Narayan *et al*., 2000). It is likely that the novel inhibitory effects of adenosine A_1 receptor stimulation reported here are mediated through changes in the activity of NHE-regulatory kinases and/or the phosphorylation status of their pertinent substrates, although this remains to be confirmed by further investigation.

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