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Specificity of action of bisindolylmaleimide protein kinase C inhibitors: do they inhibit the 70 kDa ribosomal S6 kinase in cardiac myocytes?

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Abstract

Bisindolylmaleimide protein kinase C (PKC) inhibitors, such as GF109203X and Ro31-8220, are used as pharmacological tools in many cellular systems. However, in vitro, GF109203X and Ro31-8220 also inhibit the 70 kDa ribosomal S6 kinase ($p70^{S6K}$) with similar potency. We determined whether GF109203X and Ro31-8220 inhibit $p70^{S6K}$ activity in intact adult rat ventricular myocytes (ARVM). First, we confirmed that increased phosphorylation of the 40S ribosomal S6 protein (a cellular substrate for both $p70^{S6K}$ and the 90 kDa ribosomal S6 kinase) in response to stimulation of ARVM by insulin-like growth factor-1 (300 ng/mL; 10 min) occurs specifically through rapamycin-sensitive activation of $p70^{S6K}$. Then, using this response as the index of cellular $p70^{S6K}$ activity, we determined the effects of GF109203X and Ro31-8220 (1, 3 or 10 μM) on such activity. At these concentrations, neither GF109203X nor Ro31-8220 inhibited cellular $p70^{S6K}$ activity. In contrast, even at 1 μM, cellular PKC activity (stimulated by a 3 min exposure to 30 nM phorbol 12-myristate 13-acetate) was significantly inhibited by each agent. We conclude that; (1) data obtained in vitro may not necessarily be extrapolated to intact cells and (2) inhibition of $p70^{S6K}$ is unlikely to contribute to the actions of GF109203X and Ro31-8220 in ARVM.

Keywords: Bisindolylmaleimides; Protein kinase C; Ribosomal S6 kinases; Cardiac myocytes; Drug selectivity

1. Introduction

Since the initial description of their pharmacological characteristics over a decade ago, GF109203X (bisindolylmaleimide I) [1] and Ro31-8220 (bisindolylmaleimide IX) [2] have been used extensively as ''specific'' inhibitors of protein kinase C (PKC), in order to delineate the biological functions of PKC isoforms in multiple systems. In myocardial tissue and cells, data obtained with bisindolylmaleimide inhibitors have been used to implicate PKC-mediated signalling events in the regulation of critical (patho)physiological processes, such as contractility [3], protein synthesis and myocyte hypertrophy [4] and ischemic cell death [5]. These agents have also been used to investigate the roles of PKC in the regulation of specific sarcolemmal ion transporting proteins, such as K^+ [6], Ca^{2+} [7] and Cl^- [8] channels and the Na^+/K^+ pump [9]. Additionally, experiments in our laboratory and by others have utilized bisindolylmaleimide inhibitors to explore the involvement of PKC isoforms in the stimulation of the sarcolemmal Na^+/H^+ exchanger by diverse stimuli, such as adrenergic [10,11], thrombin [12], angiotensin [13] and opioid [14] receptor agonists, anesthetic agents [15] and oxidative stress [16].

Contrary to the assumption that bisindolylmaleimides are specific inhibitors of PKC, however, it has been known for some time that, in vitro, GF109203X and Ro31-8220 also inhibit the activities of the 90 kDa ribosomal S6 kinase ($p90^{RSK}$) and the 70 kDa ribosomal S6 kinase ($p70^{S6K}$) with comparable potency [17] and, at 1 μM, can target a variety of other kinases [18]. Non-specific inhibition of $p70^{S6K}$ at PKC-inhibitory concentrations could potentially invalidate the conclusions of previous work with bisindolylmaleimide inhibitors, in particular on the roles of PKC isoforms in processes where $p70^{S6K}$ is likely to play a key

Abbreviations: ARVM, adult rat ventricular myocytes; ERK, extracellular signal-regulated kinase; IGF-1, insulin-like growth factor-1; MAPK, mitogen-activated protein kinase; MEK1, MAPK or ERK kinase 1; $p70^{S6K}$, 70 kDa ribosomal S6 kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; S6RP, 40S ribosomal protein S6.

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regulatory role (for example, in the regulation of cardiac hypertrophy [19], protein synthesis [20] and Na^+/K^+ pump activity [21]). Nevertheless, the relative effects of GF109203X and Ro31-8220 on the activities of $p70^{S6K}$ versus PKC in intact cardiac myocytes are unknown and it may not be appropriate to extrapolate from in vitro findings to the intact cell or organ.

The principal objective of the present study was to determine the effects of GF109203X and Ro31-8220 on the activity of $p70^{S6K}$ in intact ventricular myocytes isolated from the adult rat heart. To achieve this objective, we used the phosphorylation status of an endogenous substrate of $p70^{S6K}$, the 40S ribosomal protein S6 (S6RP), as an index of cellular $p70^{S6K}$ activity and determined the effects of GF109203X and Ro31-8220 on such activity that is induced by insulin-like growth factor-1 (IGF-1).

2. Materials and methods

This investigation was performed in accordance with the Home Office ''Guidance on the Operation of the Animals (Scientific Procedures) Act 1986,'' published by Her Majesty's Stationery Office, London, U.K.

2.1. Materials

Human recombinant IGF-1 was from Pharmacia and was provided as a 2 mg/mL stock solution in saline. GF109203X, Ro31-8220, PD98059 (inhibitor of mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) kinase 1 (MEK1), the upstream activator of ERK1/2) and rapamycin (inhibitor of the mammalian target of rapamycin (mTOR), the upstream activator of $p70^{S6K}$) were from Calbiochem-Novabiochem and were dissolved in DMSO to prepare stock solutions. Phorbol 12-myristate 13-acetate (PMA) was purchased from Alexis Corporation and was dissolved in 100% ethanol to prepare a stock solution. Final vehicle (DMSO or ethanol) concentration was $\leq$0.1% in any experiment and this was included in relevant control solutions. Antibodies detecting phosphorylated forms of S6RP, $p70^{S6K}$, ERK1/2 and $p90^{RSK}$ and those detecting total S6RP and $p70^{S6K}$ were from Cell Signaling Technology. Antibodies detecting total ERK2 and $p90^{RSK}$ were from Santa Cruz Biotechnology.

2.2. Isolation of ventricular myocytes

Ventricular myocytes were isolated from the hearts of adult (250–300 g) male Wistar rats (B&K Universal) by enzymatic digestion, as previously described [12]. The cell suspension (>80% rod shaped myocytes) was maintained in modified Tyrode's solution at 37 °C for 1 h, prior to use.

2.3. Determination of kinase activity

The phosphorylation status of Ser240 and Ser244 in S6RP, which are the sites targeted by $p70^{S6K}$ [22], was determined through western immunoblotting with a dual phosphospecific (pSer240/244) antibody and was used as the index of cellular $p70^{S6K}$ activity. To confirm equal protein loading, parallel western immunoblots were probed with an antibody that recognises total S6RP content. To activate $p70^{S6K}$ in a PKC-independent manner, adult rat ventricular myocytes were exposed to IGF-1 (300 ng/mL) for 0–30 min at 37 °C, in the absence or presence of kinase inhibitors, before lysis in sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (glycerol 20% (v/v), β-mercaptoethanol 3% (v/v), sodium dodecylsulphate 6% (w/v), Tris–HCl 187.5 mM, bromophenol blue 0.1 mg/mL; adjusted to pH 6.8 at room temperature with NaOH) for subsequent western immunoblotting. In some experiments, activation of ERK1/2 was assessed by determining the phosphorylation status of the Thr and Tyr residues within their regulatory Thr-Xaa-Tyr motif, by western immunoblotting with a dual phosphospecific antibody. Similarly, the activation of $p70^{S6K}$ and $p90^{RSK}$ was assessed by determining the phosphorylation status of Thr389 or Ser381, respectively, using phosphospecific antibodies. To confirm equal protein loading, parallel western immunoblots were probed with antibodies that recognise total ERK2, $p70^{S6K}$or $p90^{RSK}$ content.

2.4. Western immunoblot analysis

Protein samples in SDS-PAGE sample buffer were separated by 10–12% SDS-PAGE and subjected to western immunoblot analysis, as previously described [23]. Autoradiograms were digitised by optical scanning and quantified using NIH Image 1.62.

2.5. Experimental protocols

For the determination of drug effects on IGF-1-induced $p70^{S6K}$ activity, myocytes were incubated with each inhibitor (100 nM rapamycin, 50 μM PD98059, 1–10 μM GF109203X, 1–10 μM Ro31-8220) or vehicle (DMSO) for 15 min, prior to a 10 min stimulation with 300 ng/mL IGF-1. The reaction was stopped through the addition of SDS-PAGE sample buffer. For the determination of drug effects on PMA-induced PKC activity, myocytes were incubated with each inhibitor (1 μM GF109203X or Ro31-8220) or vehicle (DMSO) for 15 min, prior to a 3 min stimulation with 30 nM PMA or vehicle (ethanol).

2.6. Data analysis

Data (phosphorylation (arbitrary units) or the relative change in phosphorylation (%)) are expressed as mean ±

S.E.M. and were subjected to ANOVA; further analysis was by Dunnett's Test, to compare each treatment group with the relevant control. $P < 0.05$ was considered significant.

3. Results

3.1. IGF-1-induced activation of $p70^{S6K}$

Exposure of ARVM to 300 ng/mL IGF-1 increased the phosphorylation of S6RP in a time-dependent and transient manner, with the peak increase (approximately 100%) occurring after 10 min (Fig. 1). The phosphorylation of $p70^{S6K}$ was also increased in a similarly transient manner, but with an earlier peak of approximately 50% at 5 min. On the basis of these observations, a 10 min exposure to 300 ng/mL IGF-1 was selected for use as the $p70^{S6K}$-activatory stimulus in subsequent experiments. Notably, however, this stimulus also increased the phosphorylation of ERK1/2 (peak increase of approximately 150% at 2 min) and $p90^{RSK}$ (peak increase of approximately 75% at 2 min). Since $p90^{RSK}$, as well as $p70^{S6K}$, may phosphorylate S6RP [24,25], we next determined whether the IGF-1-induced increase in S6RP phosphorylation occurs through ERK-mediated activation of $p90^{RSK}$ or mTOR-mediated activation of $p70^{S6K}$. As shown in Fig. 2,

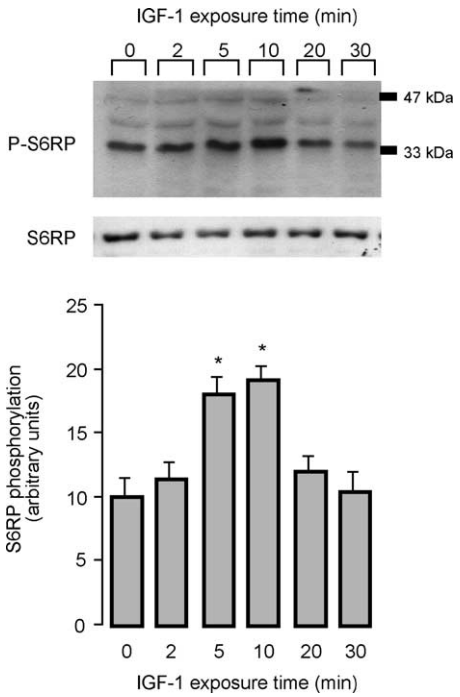

Fig. 1. Time course of IGF-1-induced S6RP phosphorylation. ARVM were exposed to 300 ng/mL IGF-1 for 0–30 min before western immunoblot analysis for phosphorylated and total S6RP (P-S6RP and S6RP, respectively). Representative immunoblot shows the detection of a prominent 33 kDa protein and the quantitative data show a significant, transient increase in S6RP phosphorylation peaking at 10 min ($^{*}P < 0.05$, $n = 6$).

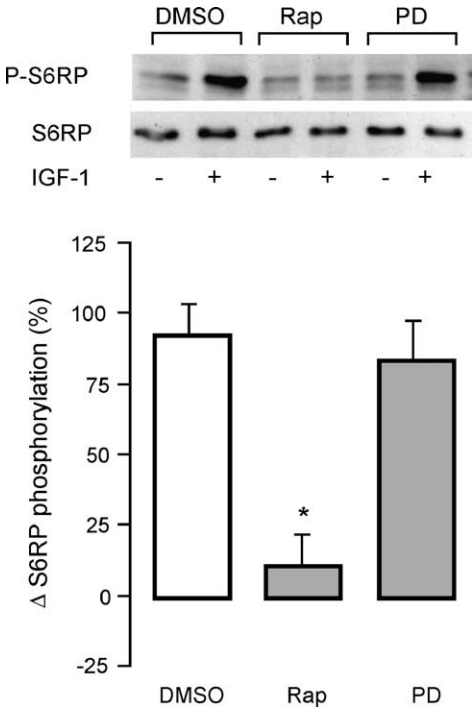

Fig. 2. Effects of PD98059 and rapamycin on IGF-1-induced S6RP phosphorylation. ARVM were pre-treated with DMSO, 100 nM rapamycin (Rap), or 50 μM PD98059 (PD), before being exposed to 300 ng/mL IGF-1 for 10 min. Representative immunoblots show the detection of phosphorylated and total S6RP (P-S6RP and S6RP, respectively) and the quantitative data illustrate significant inhibition of the IGF-1-induced increase in S6RP phosphorylation by Rap but not PD ($^{*}P < 0.05$, $n = 6$).

pre-treatment of myocytes with 100 nM rapamycin (which inhibits mTOR) abolished the IGF-1-induced increase in S6RP phosphorylation, while pre-treatment with 50 μM PD98059 (which inhibits ERK activation) had no effect. The inefficacy of PD98059 was not due to inadequate inhibition of ERK activation, since IGF-1-induced increases in the phosphorylation of ERK1/2 and $p90^{RSK}$ were both abolished by pre-treatment with this agent (data not shown). These data indicate that, in ARVM, the IGF-1-induced increase in S6RP phosphorylation occurs principally through mTOR-mediated activation of $p70^{S6K}$, such that, in this setting, the phosphorylation status of S6RP may be used as an index of cellular $p70^{S6K}$ activity.

3.2. Effects of bisindolylmaleimides on $p70^{S6K}$ activity in ARVM

At 1-10 μM, GF109203X had no significant effect on the IGF-1-induced increase in S6RP phosphorylation, while rapamycin (used as a positive control) again inhibited this response (Fig. 3A). Over the same concentration range, Ro31-8220 was similarly ineffective (Fig. 3B). These data suggest that, at concentrations $\leq$10 μM, neither GF109203X nor Ro31-8220 inhibits $p70^{S6K}$ activity in ARVM to a significant extent.

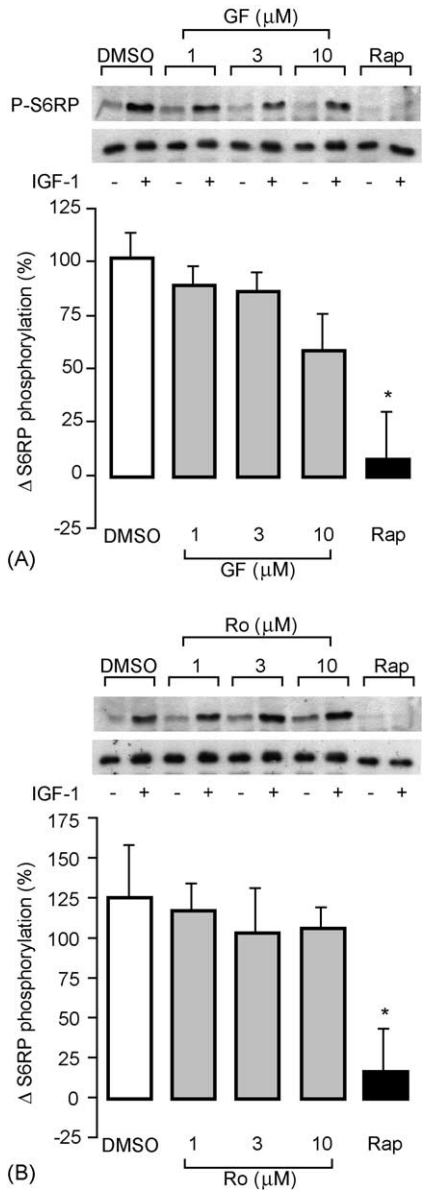

Fig. 3. Effects of (A) GF109203X and (B) Ro31-8220 on IGF-1-induced S6RP phosphorylation. ARVM were pre-treated with DMSO, 1–10 μM GF109203X (GF) or Ro31-8220 (Ro), or 100 nM rapamycin (Rap), before being exposed to 300 ng/mL IGF-1 for 10 min. Representative immunoblots show the detection of phosphorylated and total S6RP (P-S6RP and S6RP, respectively) and the quantitative data illustrate significant inhibition of the IGF-1-induced increase in S6RP phosphorylation by Rap but not GF or Ro (*$P < 0.05$, $n = 6$).

3.3. Effects of bisindolylmaleimides on PKC activity in ARVM

In order to obtain an indication of the relative selectivity of GF109203X and Ro31-8220 for PKC versus $p70^{S6K}$ in ARVM, we also determined the effects of these agents on PKC activity. Cellular PKC activity was stimulated by a 3 min exposure to 30 nM PMA, which produced a significant increase in the phosphorylation of ERK2 (Fig. 4). The PMA-induced increase in ERK2 phosphorylation was significantly inhibited by pre-treatment of cells with a

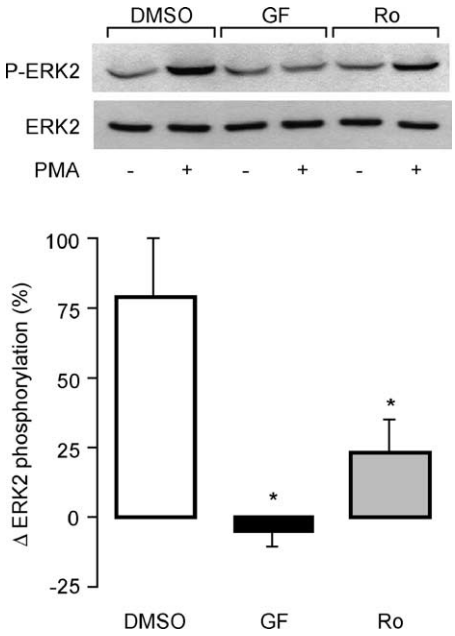

Fig. 4. Effects of GF109203X and Ro31-8220 on PMA-induced ERK phosphorylation. ARVM were pre-treated with DMSO, 1 μM GF109203X (GF), or 1 μM Ro31-8220 (Ro), before being exposed to 30 nM PMA for 3 min. Representative immunoblots show the detection of phosphorylated and total ERK2 (P-ERK2 and ERK2, respectively) and the quantitative data illustrate significant inhibition of the PMA-induced increase in ERK2 phosphorylation by both GF and Ro (*$P < 0.05$, $n = 4$).

1 μM concentration of either GF109203X or Ro31-8220 (Fig. 4), indicating that both agents produce marked inhibition of PKC activity in ARVM, even at the lowest concentration used in the present study. Therefore, in intact ARVM, GF109203X and Ro31-8220 exhibit at least 10-fold greater selectivity for PKC isoforms versus $p70^{S6K}$.

4. Discussion

Our study provides evidence that in vitro data on the specificity of GF109203X and Ro31-8220 cannot necessarily be extrapolated to the intact cell. We have demonstrated that the IGF-1-induced increase in the phosphorylation of S6RP in ARVM occurs through mTOR-mediated activation of $p70^{S6K}$. This is consistent with previous data from other cell types that $p70^{S6K}$, rather than $p90^{RSK}$, is the physiological S6RP kinase [26]. Using the IGF-1-induced increase in the phosphorylation of S6RP as an index of cellular $p70^{S6K}$ activity, we have also determined that, at concentrations $\leq$10 μM, GF109203X and Ro31-8220 do not significantly inhibit $p70^{S6K}$ activity in this cell type. It is apparent from Fig. 3, however, that GF109203X may have inhibitory effects on $p70^{S6K}$ activity at >10 μM. In contrast, even at 1 μM (a concentration used commonly in previous studies in ARVM [7,11,27]), both agents produce significant inhibition of cellular PKC activity, based on inhibition of the PMA-induced increase in ERK2 phosphorylation. Our interpretation of the data

obtained with PMA, however, is dependent on PKC activation being responsible for the increased ERK2 phosphorylation in response to this phorbol ester. It is possible that activation of other intracellular PMA receptors, such as diacylglycerol/PMA-activated Ras guanine nucleotide exchange factors [28], may contribute to this response, by inducing Ras GTP loading and downstream activation of the Raf-MEK-ERK cascade. However, there is currently no evidence that bisindolylmaleimides inhibit components of this cascade [18]. Furthermore, it is likely that the PMA-induced increase in ERK2 phosphorylation was mediated through PKC activation, since this response is inhibited by PKC downregulation [29] and heterologous expression of dominant negative PKCα [30] in neonatal rat ventricular myocytes.

The discrepancy between the lack of inhibition of $p70^{S6K}$ by GF109203X and Ro31-8220 in ARVM and the potent inhibition previously demonstrated in vitro [17,18] could potentially be explained through important differences between the in vitro and intracellular environments. Bisindolylmaleimide inhibitors exert their actions through competitive inhibition of ATP binding within the catalytic domain of PKC isoforms [1] and it is assumed that GF109203X and Ro31-8220 act in a similar manner to inhibit the catalytic activity of $p70^{S6K}$ in vitro [17]. Previous in vitro kinase assays that demonstrated potent $p70^{S6K}$ inhibition by bisindolylmaleimides were performed in the presence of an ATP concentration of 100 μM [17,18], which is approximately 50-fold lower than the estimated ATP concentration within cardiac myocytes [31]. At the higher intracellular concentration, ATP may more effectively compete with the inhibitor at their common binding site within the $p70^{S6K}$ catalytic domain. Also, in the in vitro setting, the inhibitor has unrestricted access to only the target protein of interest ($p70^{S6K}$ in this instance), in which the binding site is readily accessible. In contrast, in the intact cell, there is likely to be competition from other drug binding sites as well as modified access to some potential targets, particularly since many kinases (including activated $p70^{S6K}$ [32]) exist in multi-protein complexes. Thus, in the intact ARVM, GF109203X and Ro31-8220 may have restricted access to $p70^{S6K}$ relative to PKC isoforms, thereby exhibiting greater selectivity for the latter.

Whether bisindolylmaleimides inhibit $p70^{S6K}$ in cell types other than ARVM, or other non-PKC kinases in ARVM, is not clear. In this context, PKC-independent cellular effects of Ro31-8220 have been reported in a previous study, in which pre-treatment of rat fibroblasts with 5 μM Ro31-8220 prevented the induction of MAPK phosphatase-1 and the transcription factor c-Fos by growth factor stimulation [33]. Furthermore, Ro31-8220 was found to activate c-jun N-terminal kinase, even after PKC down-regulation, strongly suggesting a PKC-independent mechanism of action [33]. To our knowledge, such PKC-independent effects of bisindolylmaleimides have not been demonstrated in ARVM. Nevertheless, chelerythrine, a structurally-distinct PKC inhibitor, has been shown to induce apoptosis of neonatal rat ventricular myocytes through a PKC-independent mechanism [34], leading to the suggestion that PKC inhibitors may induce cellular stresses by various mechanisms [35].

The evidence to date that bisindolylmaleimide PKC inhibitors have a non-specific inhibitory effect on $p70^{S6K}$ activity relies exclusively on in vitro kinase assays. The present study shows that, at concentrations $\leq$10 μM, neither GF109203X nor Ro31-8220 significantly inhibits $p70^{S6K}$ activity in intact ARVM, suggesting that $p70^{S6K}$ inhibition is unlikely to contribute to the functional effects of these agents in this cell type.

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