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Effects of bisindolylmaleimide PKC inhibitors on p90^{RSK} activity *in vitro* and in adult ventricular myocytes

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1 Bisindolylmaleimide inhibitors of protein kinase C (PKC), such as GF109203X and Ro31-8220, have been used to investigate the roles of PKC isoforms in many cellular processes in cardiac myocytes, but these agents may also inhibit p90^{RSK} activity.

2 In *in vitro* kinase assays utilising 50 μM [ATP], GF109203X and Ro31-8220 inhibited p90^{RSK} isoforms (IC_{50} values for inhibition of RSK1, RSK2 and RSK3, respectively, were 610, 310 and 120 nM for GF109203X, and 200, 36 and 5 nM for Ro31-8220) as well as classical and novel PKC isoforms (IC_{50} values for inhibition of PKCα and PKCε, respectively, were 8 and 12 nM for GF109203X, and 4 and 8 nM for Ro31-8220).

3 At physiological [ATP] (5 mM), both GF109203X and Ro31-8220 exhibited reduced potency as inhibitors of RSK2, PKCα and PKCε (IC_{50} values of 7400, 310 and 170 nM, respectively, for GF109203X, and 930, 150 and 140 nM, respectively, for Ro31-8220), with the latter agent retaining its relatively greater potency.

4 To determine the effects of GF109203X and Ro31-8220 on p90^{RSK} activity in cultured adult rat ventricular myocytes (ARVM), phosphorylation of the eukaryotic elongation factor 2 kinase (eEF2K) at Ser366, a known p90^{RSK} target, was used as the index of such activity. Adenoviral expression of a constitutively active form of mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) kinase 1 (MEK1) was used to induce PKC-independent p90^{RSK} activation and downstream phosphorylation of eEF2K.

5 eEF2K phosphorylation was abolished by U0126 (1 μM), a selective inhibitor of MEK1, and was significantly reduced by GF109203X at $\geqslant$3 μM and by Ro31-8220 at $\geqslant$1 μM. At 1 μM, both agents inhibited PMA-induced PKC activity in ARVM.

6 These data show that GF109203X and Ro31-8220 inhibit various isoforms of PKC and p90^{RSK} *in vitro* and in intact ARVM, with the former agent exhibiting relatively greater selectivity for PKC.
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Abbreviations: ARVM, adult rat ventricular myocytes; eEF2, eukaryotic elongation factor-2; eEF2K, eEF2 kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MARCKS, myristoylated alanine-rich C-kinase substrate; MEK1, MAPK or ERK kinase 1; p90^{RSK}, 90 kDa ribosomal S6 kinase; RSK, 90 kDa ribosomal S6 kinase isoform

Introduction

Bisindolylmaleimide inhibitors of protein kinase C (PKC), such as GF109203X (bisindolylmaleimide I; originally described by Toullec *et al*., 1991) and Ro31-8220 (bisindolylmaleimide IX; originally described by Davis *et al*., 1989), have been used extensively as 'specific' inhibitors of mixed PKC isoforms, in order to delineate PKC functions in multiple systems. The use of these inhibitors, particularly GF109203X, in myocardial tissue and cells has implicated PKC-mediated signalling events in the regulation of physiological functions, such as contraction (Pi & Walker, 2000; Szokodi *et al*., 2002; von Lewinski *et al*., 2003) and protein synthesis (Ponicke *et al*., 1999), as well as a variety of pathophysiological processes, such as myocyte hypertrophy (Mullan *et al*., 1997; Ponicke *et al*., 1999; Ruf *et al*., 2002) and ischaemic cell death (Kitakaze *et al*., 1996; Chen *et al*., 1999; Inagaki *et al*., 2000; Fryer *et al*., 2001). In addition, GF109203X and Ro31-8220 have been used to investigate the roles of PKC in the regulation of sarcolemmal ion-transporting proteins, such as K^+ (Hu *et al*., 1996; Wang *et al*., 2001a), Ca^{2+} (Woo & Lee, 1999; Hu *et al*., 2000) and Cl^- (Middleton & Harvey, 1998; Duan *et al*., 1999) channels and the Na^+/K^+ pump (Jo *et al*., 2000). These inhibitors have also been utilised by this and other laboratories to explore the involvement of PKC isoforms in the stimulation of the sarcolemmal Na^+/H^+ exchanger (NHE1) by diverse stimuli, such as adrenergic (Pucéat *et al*., 1993; Snabaitis *et al*., 2000), thrombin (Yasutake *et al*., 1996), angiotensin (Gunasegaram *et al*., 1999) and opioid (Bian *et al*., 2000) receptor agonists, anaesthetic agents (Kanaya *et al*., 2001) and oxidative stress (Snabaitis *et al*., 2002). As an

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alternative to pharmacological PKC inhibition, some investigators have utilised PKC downregulation by prolonged phorbol ester stimulation (Clerk *et al*., 1998; Wang *et al*., 2001c). However, this method requires an extended period (atleast 24 h) of such stimulation and is likely to be accompanied by compensatory changes that would not occur with acute pharmacological inhibition of PKC.

Contrary to the assumption that bisindolylmaleimides are specific inhibitors of PKC, it has been known for some time that, *in vitro*, GF109203X and Ro31-8220 also inhibit the activity of the RSK2 isoform of the 90 kDa ribosomal S6 kinase ($p90^{RSK}$) family with comparable potency (Alessi, 1997). Nonspecific inhibition of $p90^{RSK}$ isoform(s) at PKC-inhibitory concentrations would complicate the interpretation of data obtained with bisindolylmaleimide inhibitors. This would be of particular concern when investigating the roles of PKC isoforms in (patho)physiological processes, such as the regulation of NHE1 activity (Takahashi *et al*., 1999; Snabaitis *et al*., 2000; Haworth *et al*., 2003) and protein synthesis (Wang, 2001b; Wang & Proud, 2002) and the pathogenesis of heart failure (Takeishi *et al*., 2002; Aker *et al*., 2004)), where $p90^{RSK}$ activity is also likely to play a role. Nevertheless, the relative effects of GF109203X and Ro31-8220 on the activities of the different isoforms of $p90^{RSK}$ *versus* PKC *in vitro* and in cardiac myocytes are unknown, and it may not be appropriate to extrapolate from *in vitro* findings with RSK2 to the intact cell or organ. In this context, a previous study from our laboratory has indicated that, in contrast to *in vitro* findings (Alessi, 1997), GF109203X and Ro31-8220 do not inhibit the 70 kDa ribosomal S6 kinase (p70S6K) in intact adult rat ventricular myocytes (ARVM) (Roberts *et al*., 2004).

In this study, we determined: (1) the relative *in vitro* potencies of GF109203X and Ro31-8220 as inhibitors of recombinant $p90^{RSK}$ isoforms RSK1, RSK2 and RSK3 *versus* recombinant PKC isoforms PKCα and PKCε; (2) the *in vitro* selectivity of these bisindolylmaleimide inhibitors for recombinant PKC isoforms *versus* recombinant RSK2, the predominant $p90^{RSK}$ isoform in myocardium, at a physiological concentration of ATP; (3) the concentration-dependent effects of GF109203X and Ro31-8220 on the total cellular activities of native $p90^{RSK}$ *versus* PKC isoforms expressed in intact ARVM.

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.

Synthesis and purification of recombinant proteins

Bacterial expression vectors encoding GST-NHE1 and GST-MARCKS (pGEX-KG and pGEX-2 T, respectively) were transformed into the BL21 strain of *Escherichia coli*. Cultures were grown to the sub-log phase and induced for 3 h at 37°C in 0.5 mM isopropyl-β-D-thioglactopyranoside. Cells were then harvested, resuspended in phosphate-buffered saline (PBS) containing 1% v v^{-1} Triton X-100 (PBS-T), sonicated on ice, and centrifuged at 12,000 r.p.m. for 30 min at 4°C. The fusion protein, contained in the supernatant, was passed through a pre-packed glutathione-sepharose 4B column (PharmaciaBiotech) at 4°C. Bound fusion protein was then washed extensively with PBS-T before being eluted from the column using reduced glutathione (5 mM) in Tris-HCl (50 mM) at pH 8.0. The eluted sample was concentrated using a Centriplus 30 kDa filter device (Millipore) until sample volume was approximately 500 μl. Protein concentration of the sample was then determined using a Bradford assay, and the presence of the pertinent fusion protein was confirmed by Coomassie blue staining after protein separation by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Determination of kinase activity in vitro

GST-NHE1 and GST-MARCKS were used as substrates for recombinant human $p90^{RSK}$ isoforms (RSK1, RSK2, RSK3) and recombinant human PKCα and PKCε, respectively, in *in vitro* kinase assays. Serial dilutions of GF109203X and Ro31-8220 (1 nM–10 μM) and dilutions of all other reagents were performed in kinase assay buffer (Tris-base 1 M, $MgCl_2$ 1 M, DTT 1 M; pH 7.6). Aliquots (40 μl) of the assay mixture (containing protein kinase (0.1 U ml^{-1}), protein kinase substrate (2 μM), and either GF109203X or Ro31-8220 (1 nM–10 μM) were stored on ice. The phosphorylation reaction was started by the addition of 10 μl kinase assay buffer containing ATP (50 μM or 5 mM). In experiments using PKCα or PKCε, phosphatidylserine (50 μg ml^{-1}), diacylglycerol (5 μg ml^{-1}) and $CaCl_2$ (100 μM) were also present during the reaction. The aliquots were incubated for 15 min at 37°C before the reaction was stopped by the addition of SDS–PAGE sample buffer (glycerol 20% v v^{-1}, β-mercaptoethanol 3% v v^{-1}, sodium dodecylsulphate 6% w v^{-1}, Tris-HCl 187.5 mM, bromophenol blue 0.1 mg ml^{-1}; pH 6.8) and samples were stored at −20°C for subsequent Western immunoblot analysis.

Phosphorylation status of protein kinase substrates was determined through Western immunoblot analysis using phosphospecific antibodies recognising the sequence RXRXX(pS), to detect $p90^{RSK}$-induced phosphorylation of GST-NHE1, or raised against a peptide comprising pS152/pS156 of MARCKS, to detect PKC-induced phosphorylation of GST-MARCKS. Equal substrate loading was confirmed through Western immunoblot analysis using an antibody recognising GST. Each experiment was repeated 3–6 times.

Western immunoblot analysis

Protein samples in SDS–PAGE sample buffer (20 μl of reaction mix from *in vitro* kinase assays; 20 μl of cellular extract (approximately 20 μg protein) from cultured ARVM) were subjected to SDS–PAGE (8 or 12%) and transferred to PVDF membranes. The membranes were incubated for 2 h, at room temperature, in Tris-buffered saline (TBS) containing 0.1% v v^{-1} Tween20 (TBS-T) and 10% w v^{-1} non-fat dry milk. They were then washed several times in TBS-T and incubated with primary antibody (in TBS-T containing 1% w v^{-1} nonfat dry milk) overnight at 4°C on an orbital shaker. Following three 10-min washes in TBS-T, the membranes were incubated with horseradish peroxidase-coupled anti-rabbit IgG secondary antibody for 1 h at room temperature. Membranes were then washed three times (3 × 15 min) in TBS-T, before incubation with enhanced chemiluminescence reagent. Bands were visualised by exposure of the membranes to X-ray film,

digitised by optical scanning and quantified using NIH Image 1.62.

Isolation of ARVM

ARVM were isolated from the hearts of adult (250–300 g body weight) male Wistar rats (B&K Universal, Hull, U.K.) by enzymatic digestion, as described previously (Snabaitis *et al*., 2005). In brief, rats were anaesthetised with sodium pentobarbitone (100 mg kg^{-1} i.p.) and hearts were excised and perfused (37°C) in the Langendorff mode for three sequential periods, as follows: (1) with modified Tyrode's solution (in mM: NaCl 130, KCl 5.4, $MgCl_2$ 1.4, NaH_2PO_4 0.4, taurine 20, creatinine 10, HEPES 10, and glucose 10; adjusted to pH 7.3 at 37°C with NaOH) containing 0.75 mM $CaCl_2$, for 5 min, (2) with nominally calcium-free modified Tyrode's solution containing 0.1 mM EGTA, for 4 min, and (3) with modified Tyrode's solution containing 0.1 mM $CaCl_2$ and collagenase (Worthington type II, 0.75 mg ml^{-1}), for 8 min. All solutions were gassed with 100% O_2 and coronary flow rate was maintained at 12 ml min^{-1}. After the perfusion protocol, the ventricles were removed and chopped into several pieces in modified Tyrode's solution containing 0.1 mM $CaCl_2$ and 0.75 mg ml^{-1} collagenase. The tissue fragments were then gently agitated and bubbled with 100% O_2 for 5 min to facilitate cell dispersion before being allowed to settle by gravity. The calcium concentration of modified Tyrode's solution was increased in two steps to 1 mM and the cells kept at room temperature for subsequent culture.

Short-term culture and adenoviral infection of ARVM

ARVM were washed in M199 medium (Invitrogen, Paisley, U.K.) with added penicillin (100 i.u. ml^{-1}) and streptomycin (100 i.u. ml^{-1}). The cell suspension was centrifuged at $100 \times g$ for 2 min to pellet the myocytes, which were then resuspended in modified M199 (mM199) medium (M199 medium with added penicillin (100 i.u. ml^{-1}), streptomycin (100 i.u. ml^{-1}), L-carnitine (2 mM), creatine (5 mM) and taurine (5 mM)). To each well of a laminated six-well culture plate, 2 ml of cell suspension was added and the plates were maintained in a 5% CO_2 incubator at 37°C. After 2 h of pre-plating, the medium was aspirated, leaving only adherent cells, and 2 ml of fresh, pre-warmed mM199 medium was added.

Adenoviral infection of cultured myocytes was performed after the initial 2 h pre-plating step. The number of rod-shaped cells in a field of 1 mm^2 (as defined by an eye-piece graticule) was counted in several wells and used to estimate the number of cells per well. Myocytes were exposed to adenovirus encoding constitutively active MEK1 (caMEK1) at a multiplicity of infection (MOI) of 0–1000 plaque forming units (PFU)/cell for 1 h at 37°C, before the medium containing residual virus was removed by aspiration and replaced with fresh, pre-warmed (37°C) mM199 medium. Experiments were performed 42 h after adenoviral infection.

Determination of cellular kinase activity in ARVM

The phosphorylation status of S366 in eEF2K, the site targeted by $p90^{RSK}$ (Wang *et al*., 2001b), was determined through Western immunoblotting with a phosphospecific antibody and was used as the index of cellular $p90^{RSK}$ activity. To activate native $p90^{RSK}$ isoforms in a PKC-independent manner, ARVM were infected with adenovirus encoding caMEK1 and treated with either a kinase inhibitor (U0126, Rapamycin, GF109203X or Ro31-8220) or vehicle, before being lysed in SDS–PAGE sample buffer for subsequent Western immunoblot analysis.

Adenoviral expression of caMEK1 was determined through Western immunoblotting with an MEK1 antibody. Downstream activation of ERK1/2 was assessed by determining the phosphorylation status of the T and Y residues within their regulatory T–X–Y motif, by Western immunoblotting with a dual phosphospecific antibody. Similarly, the activation of $p90^{RSK}$ and the phosphorylation status of eEF2 were assessed by Western immunoblotting with phosphospecific antibodies that recognise phosphorylated S381 or T56, respectively. To confirm equal protein loading throughout, parallel Western immunoblots were probed with an antibody that recognises total ERK2. To determine PKC activity in ARVM, the phosphorylation status of the PKC substrate PKD was assessed by Western immunoblotting with a phosphospecific antibody that recognises phosphorylated S916.

Experimental protocols

For the determination of drug effects on $p90^{RSK}$ activity, ARVM were infected with adenovirus encoding caMEK1 (50 PFU $cell^{-1}$). At 42 h after infection, cells were incubated with a kinase inhibitor (U0126 (1 μM), rapamycin (100 nM), GF109203X (1–10 μM), Ro31-8220 (1–10 μM)) or vehicle (DMSO) for 4 h, prior to the addition of SDS–PAGE sample buffer. For the determination of drug effects on PKC activity, ARVM were pretreated with a kinase inhibitor (GF109203X (1 μM) or Ro31-8220 (1 μM)) or vehicle (DMSO) for 15 min, prior to a 5-min stimulation with 30 nM PMA or vehicle (ethanol) and subsequent lysis in SDS–PAGE sample buffer. Each experiment was repeated 3–6 times.

Data analysis

All data are expressed as mean ± s.e.m. Dose–response curves and IC_{50} values were obtained by nonlinear regression analysis of *in vitro* phosphorylation data, using GraphPad Prism 4 software. Data on *in vivo* phosphorylation (arbitrary units) or the relative change in phosphorylation (%) were subjected to ANOVA; further analysis was performed using Dunnett's test (to compare each treatment group with a single control) or Student–Newman–Keuls test (for multiple comparisons). $P < 0.05$ was considered significant.

Materials

Recombinant active human PKC and $p90^{RSK}$ isoforms were from Upstate Biotechnology. Plasmids encoding glutathione S-transferase (GST)-linked fusion proteins comprising the full-length myristoylated alanine-rich C-kinase substrate (MARCKS) protein (GST-MARCKS) or amino acids 625–747 of NHE1 (GST-NHE1) were kind gifts from Dr T. Herget (Johannes Gutenberg University, Germany) (Herget & Rozengurt, 1994) and Dr B. Berk (University of Rochester, U.S.A.) (Takahashi *et al*., 1997), respectively. Adenovirus

encoding a constitutively active form of mitogen-activated protein kinase (MAPK) or extracellular signal-regulated kinase (ERK) kinase 1 (MEK1), the upstream activator of ERK1/2, was a kind gift from Dr J. Molkentin (Cincinnati Children's Hospital Medical Centre, U.S.A.) (Bueno *et al*., 2000). GF109203X, Ro31-8220, U0126 (an inhibitor of MEK1) and rapamycin (an inhibitor of the mammalian target of rapamycin (mTOR)) were from Merck Biosciences, Nottingham, U.K. and were dissolved in DMSO to prepare stock solutions. Final vehicle (DMSO) concentration was $\leqslant 0.1\%$ in any experiment and this was included in relevant control solutions. Antibodies detecting phosphorylated forms of eukaryotic elongation factor-2 (eEF2), eEF2 kinase (eEF2K), ERK1/2, $p90^{RSK}$, protein kinase D (PKD), NHE-1 and MARCKS, and antibody detecting total MEK1 were from Cell Signalling Technology, Hertfordshire, U.K. Antibody detecting total ERK2 was from Santa Cruz Biotechnology, California, U.S.A. and antibody detecting GST was from Amersham Biosciences, Buckinghamshire, U.K.

Results

Effects of bisindolylmaleimides on PKC and $p90^{RSK}$ isoform activity in vitro

Recombinant human PKC isoforms PKCα and PKCε induced a time-dependent phosphorylation of MARCKS, with the reaction reaching saturation after approximately 45 min under our conditions (Figure 1a). Similarly, recombinant human $p90^{RSK}$ isoforms RSK1, RSK2 and RSK3 induced a time-dependent phosphorylation of the fusion protein comprising NHE1 amino acids 625–747, with maximum phosphorylation occurring after approximately 30 min (Figure 1b). On the basis that, with extended reaction times, even a reduced kinase activity would produce complete phosphorylation of the available substrate, a 15-min reaction time, which produced substantial but submaximal substrate phosphorylation, was selected for use in subsequent *in vitro* kinase activity assays designed to determine the inhibitory effects of bisindolylmaleimides on PKC and $p90^{RSK}$ isoform activities.

As expected, at a low ATP concentration (50 μM), GF109203X and Ro31-8220 inhibited both PKCα and PKCε with high potency, with no apparent isoform selectivity (Figure 2a and b, top panels; Table 1). Both bisindolylmaleimides also inhibited all three $p90^{RSK}$ isoforms, in a dose-dependent manner (Figure 2a and b, bottom panels; Table 1). GF109203X exhibited a rank order of potency against $p90^{RSK}$ isoforms of RSK3 > RSK2 > RSK1, with approximately two- to five-fold differences in IC_{50} values for different isoforms (Table 1). Ro31-8220 exhibited the same rank order of potency as GF109203X but a greater degree of selectivity between $p90^{RSK}$isoforms, with approximately six- to 40-fold differences in IC_{50} values for RSK1, RSK2 and RSK3 (Table 1).

We also determined the *in vitro* potency of GF109203X and Ro31-8220 as inhibitors of PKCα, PKCε and RSK2 (the predominant $p90^{RSK}$ isoform expressed in myocardium (Wagner, 2004)) at an ATP concentration (5 mM) that is akin to the estimated intracellular ATP concentration in ARVM (Allue *et al*., 1996). At this ATP concentration, the inhibitory potencies of both bisindolylmaleimides against all three

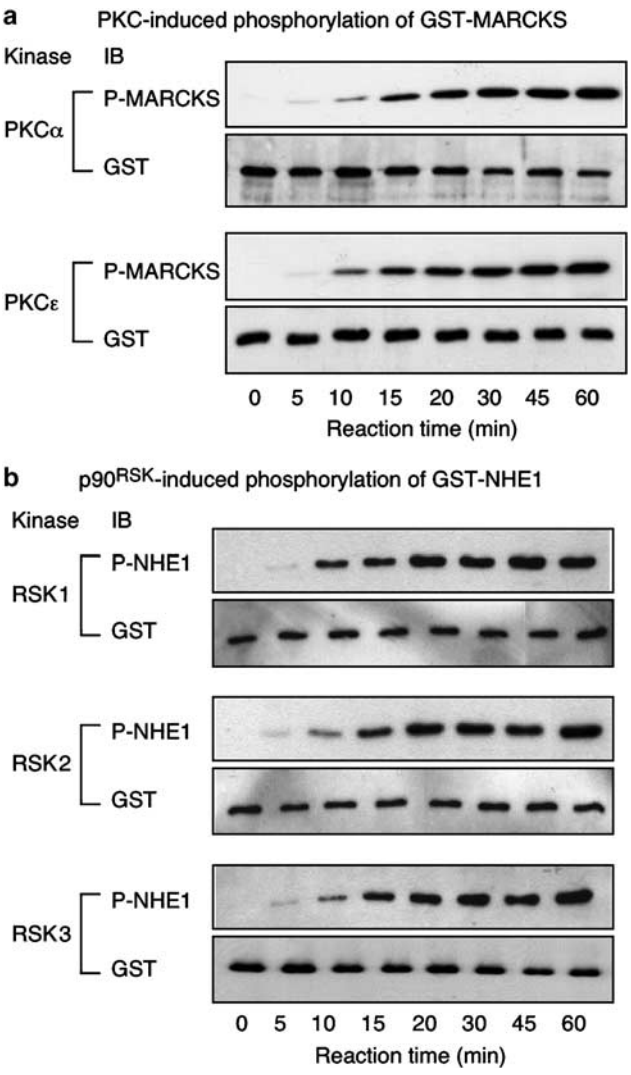

Figure 1 Time-dependent phosphorylation of (a) GST-MARCKS by the PKC isoforms PKCα and PKCε and (b) GST-NHE1 by the $p90^{RSK}$ isoforms RSK1, RSK2 and RSK3. Recombinant human PKCα and PKCε were incubated with GST-MARCKS for 0–60 min at 37°C, prior to addition of SDS–PAGE sample buffer and Western immunoblot analysis with an antibody recognising pS152/pS156 of MARCKS. Similarly, recombinant human RSK1, RSK2 and RSK3 were incubated with GST-NHE1 for 0–60 min at 37°C, prior to addition of SDS–PAGE sample buffer and Western immunoblot analysis with an antibody recognising the RXRXX(pS) motif in GST-NHE1. An antibody recognising GST was used to confirm the presence of equal amounts of substrate. Autoradiograms representative of three experiments.

Table 1 Inhibitory potencies of GF109203X and Ro31-8220

	IC_{50} (nM)			
	50 μM [ATP]		*5 mM [ATP]*	
Kinase	*GF109203X*	*Ro31-8220*	*GF109203X*	*Ro31-8220*
PKCα	8	4	310	150
PKCε	12	8	170	140
RSK1	610	200	—	—
RSK2	310	36	7400	930
RSK3	120	5	—	—

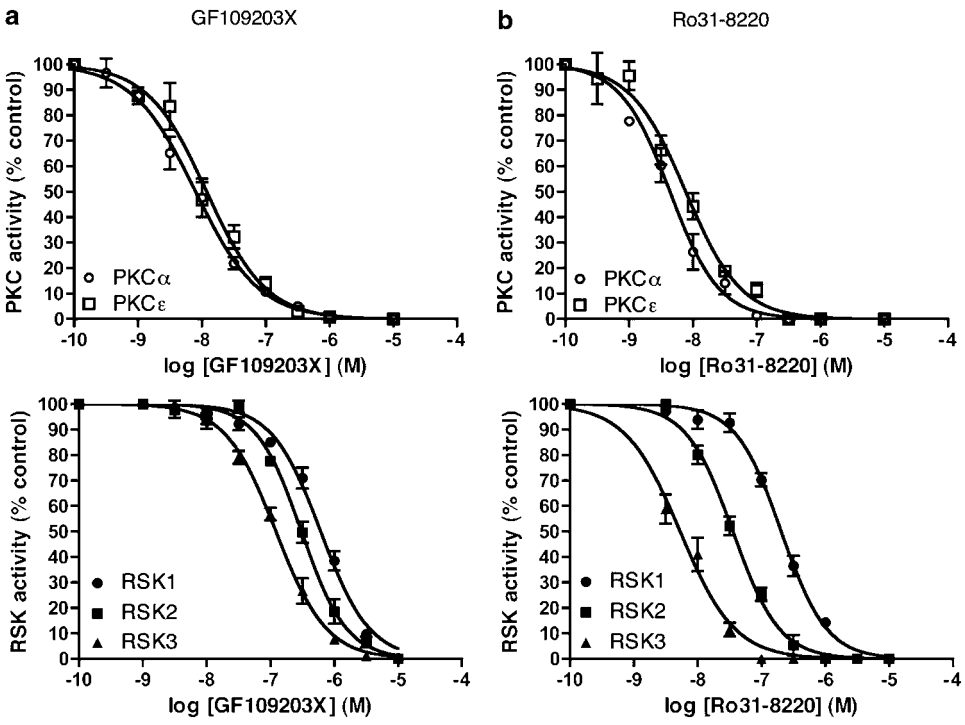

Figure 2 Concentration-dependent inhibition of PKC and p90^{RSK} isoforms *in vitro* by (a) GF109203X and (b) Ro31-8220, in the presence of low [ATP] (50 μM). Recombinant human PKCα and PKCε were incubated with GST-MARCKS and either GF109203X or Ro31-8220 (1 nM–10 μM) for 15 min at 37°C. Reactions were stopped in SDS–PAGE sample buffer and samples underwent Western immunoblot analysis with an antibody recognising pS152/pS156 of MARCKS (top panels). Similarly, recombinant human RSK1, RSK2 and RSK3 were incubated with GST-NHE1 and either GF109203X or Ro31-8220 (1 nM–10 μM) for 15 min at 37°C. Reactions were stopped in SDS–PAGE sample buffer and samples underwent Western immunoblot analysis with an antibody recognising the RXRXX(pS) motif in GST-NHE1 (bottom panels). $n=4$ experiments.

kinases were markedly reduced (Figure 3a and b; Table 1). Interestingly, GF109203X exhibited greater selectivity for the PKC isoforms, with the IC_{50} value for RSK2 inhibition being 24- and 43-fold greater than the corresponding values for PKCα and PKCε inhibition, respectively (Table 1). In comparison, for Ro31-8220, the IC_{50} value for RSK2 inhibition was only six-fold greater than those for PKCα and PKCε inhibition (Table 1).

p90^{RSK} activation in intact ARVM by adenoviral expression of caMEK1

Infection of ARVM with adenovirus encoding caMEK1 resulted in an MOI-dependent increase in MEK1 expression (Figure 4). Constitutive activity of the heterologously expressed protein gave rise to an MOI-dependent increase in the phosphorylation status of the downstream components of this canonical MAPK pathway. Thus, increased phosphorylation of ERK1/2, p90^{RSK} and eEF2K was detectable at the lowest MOI of 10 PFU cell^{-1} and was statistically significant at an MOI of 50 PFU cell^{-1} (Figure 4). Since phosphorylation of eEF2K results in its inactivation (Wang *et al*., 2001b), heterologous expression of caMEK1 also resulted in an MOI-dependent reduction in the phosphorylation of eEF2, the eEF2K substrate (Figure 4).

It is known that p70^{S6K}, as well as p90^{RSK}, may phosphorylate eEF2K at a common site that is detected by the phosphospecific antibody used (Wang *et al*., 2001b). Therefore, we next determined whether the increased phosphorylation of eEF2K that arises from heterologous expression of caMEK1 occurs solely through MEK1-mediated activation of p90^{RSK}, or whether mTOR-mediated activation of p70^{S6K} also plays a role. As shown in Figure 5, increased phosphorylation of eEF2K in ARVM infected with the caMEK1 adenovirus (50 PFU cell^{-1}) was inhibited by the MEK inhibitor U0126, which also inhibited the increased phosphorylation of ERK1/2 and p90^{RSK} and the decreased phosphorylation of eEF2. In contrast, the mTOR inhibitor rapamycin had no effect on these cellular responses to heterologous expression of caMEK1, including the increase in eEF2K phosphorylation (Figure 5). Consistent with a recent report (Wang & Proud, 2002), these data indicate that, in ARVM with heterologous expression of caMEK1, increased eEF2K phosphorylation occurs principally through p90^{RSK}activity. Thus, in this setting, the phosphorylation status of eEF2K may be used as an index of such activity.

Effects of bisindolylmaleimides on p90^{RSK} activity in intact ARVM

At 1–10 μM, GF109203X had no significant effect on the increases in ERK1/2 or p90^{RSK} phosphorylation induced by heterologous expression of caMEK1, while U0126 (used as a positive control) again inhibited these responses (Figure 6, panels a–c), as well as the increase in eEF2K phosphorylation (Figure 6, panel d) and the decrease in eEF2 phosphorylation

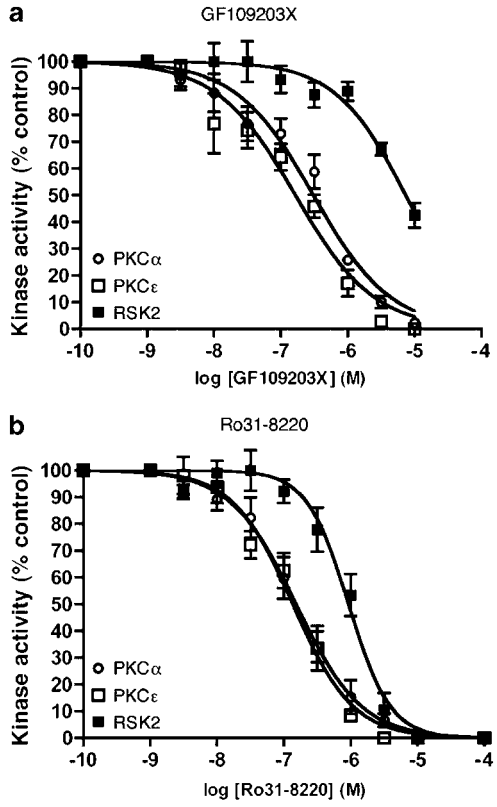

Figure 3 Concentration-dependent inhibition of PKC isoforms and RSK2 *in vitro* by (a) GF109203X and (b) Ro31-8220, in the presence of physiological [ATP] (5 mM). Recombinant human PKCα and PKCε were incubated with GST-MARCKS and either GF109203X or Ro31-8220 (1 nM–10 μM) for 15 min at 37°C. Reactions were stopped in SDS–PAGE sample buffer and samples underwent Western immunoblot analysis with an antibody recognising pS152/pS156 of MARCKS. Similarly, recombinant human RSK2 was incubated with GST-NHE1 and either GF109203X or Ro31-8220 (1 nM–10 μM) for 15 min at 37°C. Reactions were stopped in SDS–PAGE sample buffer and samples underwent Western immunoblot analysis with an antibody recognising the RXRXX(pS) motif in GST-NHE1. $n=4$ experiments.

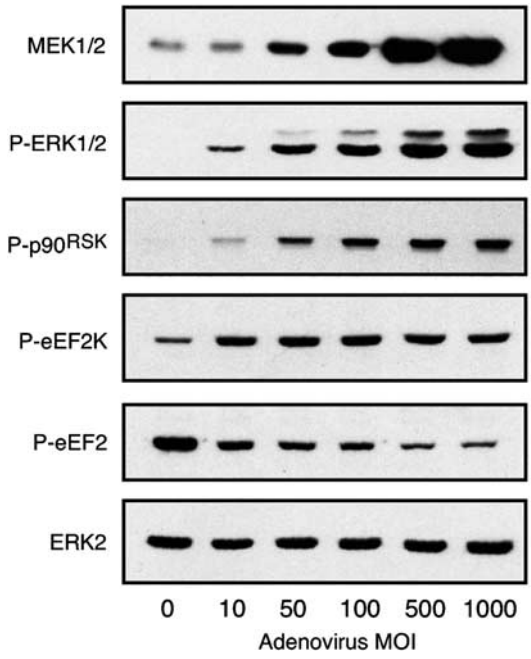

Figure 4 Activation of the ERK/p90^{RSK} pathway by adenoviral expression of caMEK1. ARVM were maintained in culture for 42 h, following a 1 h infection with adenovirus encoding caMEK1 at MOI of 10–1000 PFU cell^{-1}. Cellular protein samples were subsequently subjected to Western immunoblot analysis for expression of MEK1 and phosphorylated forms of ERK1/2 (P-ERK1/2), p90^{RSK}(P-p90^{RSK}), eEF2K (P-eEF2K) and eEF2 (P-eEF2). Total ERK2 expression is also shown to illustrate equal protein loading. Autoradiograms representative of six experiments.

(Figure 6, panel e). At concentrations $\geqslant 3\,\mu$M, GF109203X also significantly inhibited the increase in eEF2K phosphorylation, such that with the 10 μM concentration eEF2K phosphorylation was no longer significantly different from that in uninfected control cells (Figure 6, panel d). Although the increase in eEF2K phosphorylation was abolished by 10 μM GF109203X, a parallel inhibition of the reduction in eEF2 phosphorylation was not observed (Figure 6, panel e). Over the same concentration range, Ro31-8220 was similarly ineffective against the changes in the phosphorylation status of ERK1/2, p90^{RSK} and eEF2 that arose from the heterologous expression of caMEK1 (Figure 7, panels a–c, e). However, like GF109203X, Ro31-8220 caused a concentration-dependent reduction in eEF2K phosphorylation (Figure 7, panel d). Indeed, Ro31-8220 was considerably more potent than GF109203X as an inhibitor of eEF2K phosphorylation, such that even the lowest concentration of 1 μM produced a significant reduction in the response to the heterologous expression of caMEK1 (Figure 7, panel d). These data suggest that GF109203X and Ro31-8220 both significantly inhibit p90^{RSK} activity in intact ARVM, at concentrations $\geqslant 3$ and $\geqslant 1\,\mu$M, respectively.

Effects of bisindolylmaleimides on PKC activity in intact ARVM

To obtain an indication of the relative selectivity of GF109203X and Ro31-8220 for native PKC *versus* p90^{RSK} isoforms in intact ARVM, we also determined the effects of these inhibitors on cellular PKC activity. The activation status of the downstream PKC substrate PKD, determined by Western immunoblotting using a phosphospecific antibody (Haworth *et al*., 2000), was used as the index of cellular PKC activity. Stimulation of cellular PKC activity by PMA produced a significant increase in PKD phosphorylation (Figure 8). The PMA-induced increase in PKD phosphorylation was significantly reduced by a 1 μM concentration of either GF109203X or Ro31-8220 (Figure 8), indicating that cellular PKC activity is inhibited by both agents, even at the lowest concentration used in the present study.

Discussion

Our study demonstrates, for the first time to our knowledge, that all three p90^{RSK} isoforms (RSK1, RSK2 and RSK3) are inhibited by GF109203X and Ro31-8220 *in vitro*, and that, even at a physiological ATP concentration, both bisindolyl-

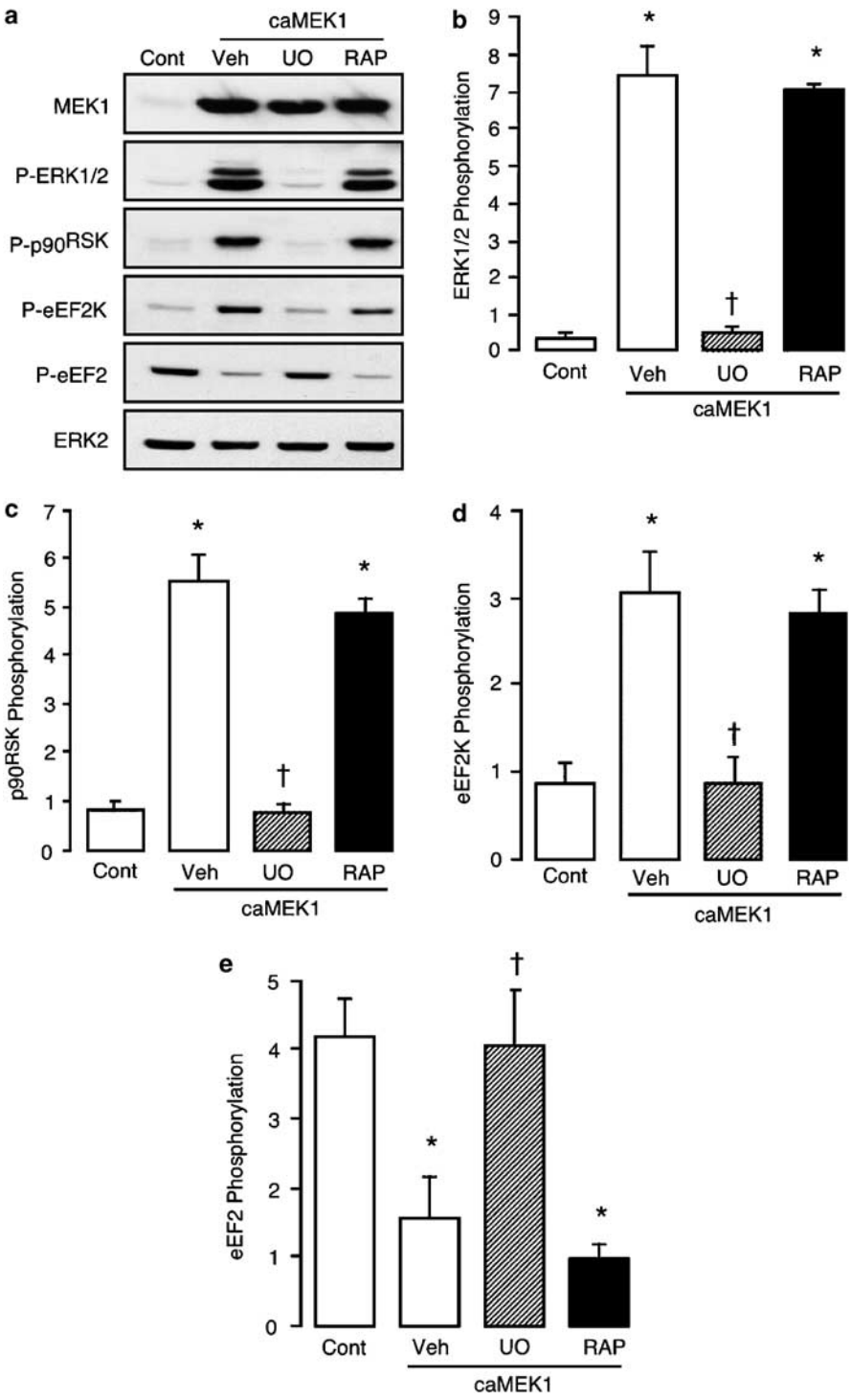

Figure 5 Effects of U0126 and rapamycin on the phosphorylation status of ERK1/2, $p90^{RSK}$, eEF2K and eEF2 following adenovirus-mediated expression of caMEK1. ARVM were maintained in culture for 42 h, following a 1 h infection with empty virus (Cont) or adenovirus encoding caMEK1, both at an MOI of 50 PFU cell^{-1}. ARVM were then exposed to vehicle (Veh), 1 μM U0126 (UO) or 100 nM rapamycin (RAP) for 4 h, before being lysed in SDS–PAGE sample buffer for subsequent Western immunoblot analysis. (a) Representative Western immunoblots showing the expression of MEK1 and phosphorylated forms of ERK1/2 (P-ERK1/2), $p90^{RSK}$(P-$p90^{RSK}$), eEF2K (P-eEF2K) and eEF2 (P-eEF2). Total ERK2 expression is also shown to illustrate equal protein loading. Quantitative data (panels b–e) illustrate the phosphorylation status of (b) ERK1/2, (c) $p90^{RSK}$, (d) eEF2K and (e) eEF2, $^{*}P<0.05$ *versus* Cont, $^{\dagger}P<0.05$ *versus* Veh ($n=6$).

maleimides retain their inhibitory effects on RSK2 (the predominant $p90^{RSK}$ isoform in cardiac myocytes (Wagner, 2004)). Furthermore, the study provides novel data which indicate that GF109203X and Ro31-8220 significantly inhibit $p90^{RSK}$ activity in the intact ARVM at concentrations $\geqslant 3$ and $\geqslant 1$ μM, respectively.

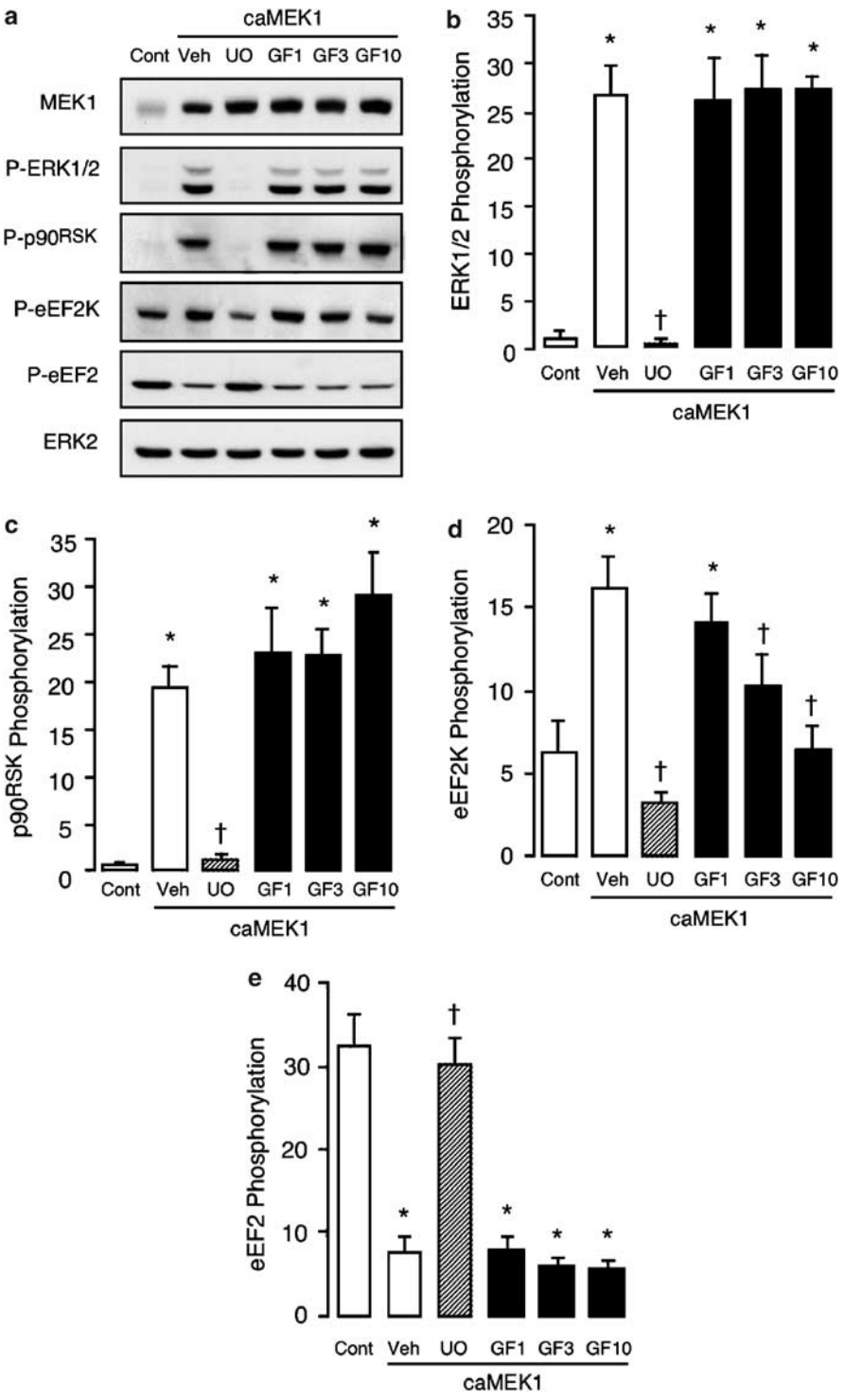

Figure 6 Effects of GF109203X on the phosphorylation status of ERK1/2, $p90^{RSK}$, eEF2K and eEF2 following adenoviral expression of caMEK1. ARVM were maintained in culture for 42 h, following a 1 h infection with empty virus (Cont) or adenovirus encoding caMEK1, both at an MOI of 50 PFU cell^{-1}. ARVM were then exposed to vehicle (Veh), 1 μM U0126 (UO) or 1–10 μM GF109203X (GF) for 4 h, before being lysed in SDS–PAGE sample buffer for subsequent Western immunoblot analysis. (a) Representative Western immunoblots showing the expression of MEK1 and phosphorylated forms of ERK1/2 (P-ERK1/2), $p90^{RSK}$(P-$p90^{RSK}$), eEF2K (P-eEF2K) and eEF2 (P-eEF2). Total ERK2 expression is also shown to illustrate equal protein loading. Quantitative data (panels b–e) illustrate the phosphorylation status of (b) ERK1/2, (c) $p90^{RSK}$, (d) eEF2K and (e) eEF2, $^{*}P<0.05$ *versus* Cont, $^{\dagger}P<0.05$ *versus* Veh ($n=6$).

Our *in vitro* data extend the observations of Alessi (1997), who reported the inhibitory effects of GF109203X and Ro31-8220 on the activities of mixed PKC isoforms purified from rat brain and RSK2 (also known as MAPKAP kinase-1β) purified from rabbit skeletal muscle. In the present work, the effects of these agents on the activities of representative recombinant

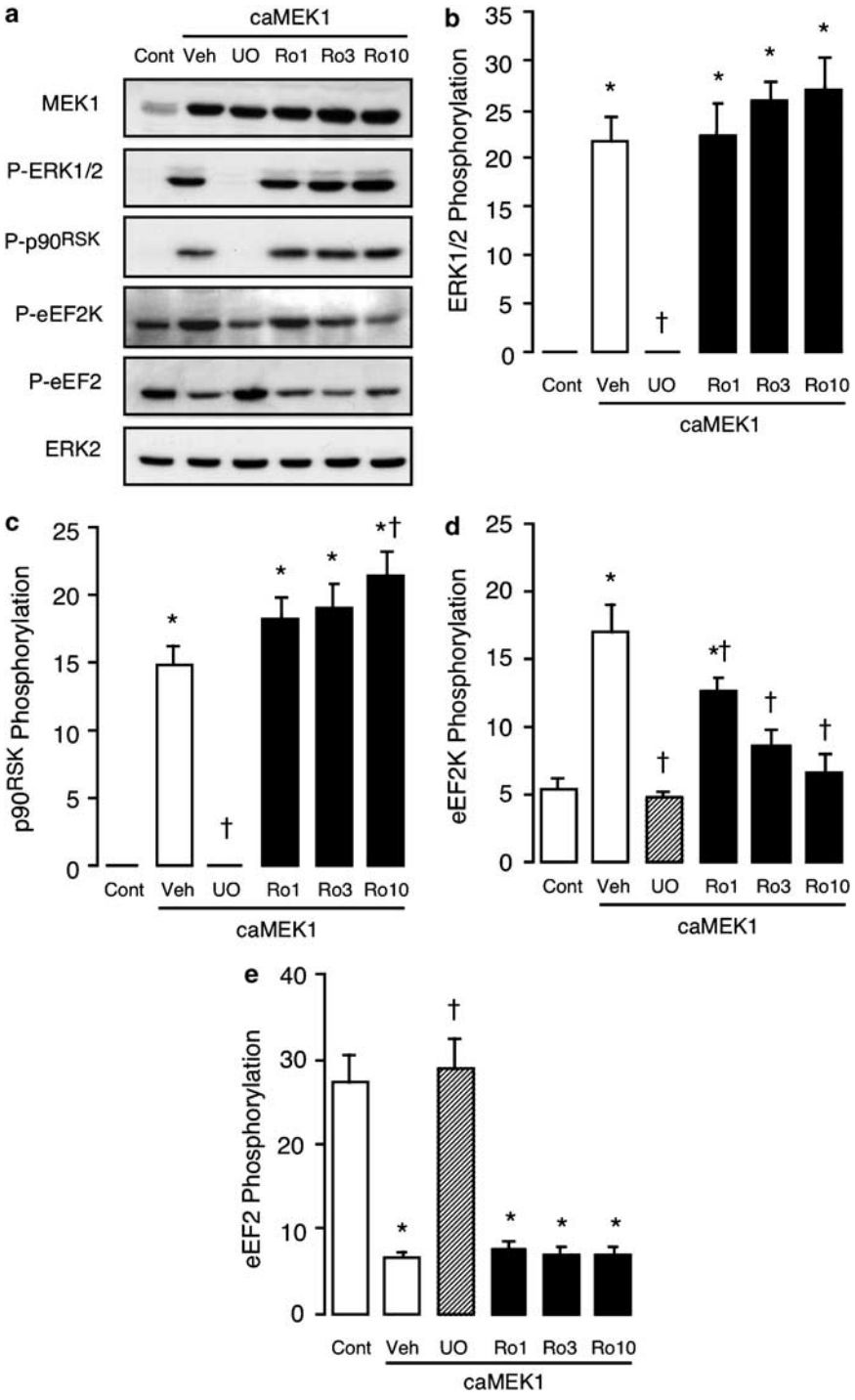

Figure 7 Effects of Ro31-8220 on the phosphorylation status of ERK1/2, $p90^{RSK}$, eEF2K and eEF2 following adenoviral expression of caMEK1. ARVM were maintained in culture for 42 h, following a 1 h infection with empty virus (Cont) or adenovirus encoding caMEK1, both at an MOI of 50 PFU cell^{-1}. ARVM were then exposed to vehicle (Veh), 1 μM U0126 (UO) or 1–10 μM Ro31-8220 (Ro) for 4 h, before being lysed in SDS–PAGE sample buffer for subsequent Western immunoblot analysis. (a) Representative Western immunoblots showing the expression of MEK1 and phosphorylated forms of ERK1/2 (P-ERK1/2), $p90^{RSK}$(P-$p90^{RSK}$), eEF2K (P-eEF2K) and eEF2 (P-eEF2). Total ERK2 expression is also shown to illustrate equal protein loading. Quantitative data (panels b–e) illustrate the phosphorylation status of (b) ERK1/2, (c) $p90^{RSK}$, (d) eEF2K and (e) eEF2, $^{*}P<0.05$ *versus* Cont, $^{\dagger}P<0.05$ *versus* Veh ($n=6$).

human PKC isoforms from the classical and novel PKC subfamilies (PKCα and PKCε, respectively), as well as the recombinant human $p90^{RSK}$ isoforms RSK1, RSK2 and RSK3 were determined. Additionally, the impact of increasing the ATP concentration to a physiological level on the inhibitory potencies of these agents against the selected PKC isoforms and RSK2 was examined. All three $p90^{RSK}$ family isoforms studied were inhibited by both GF109203X and Ro31-8220

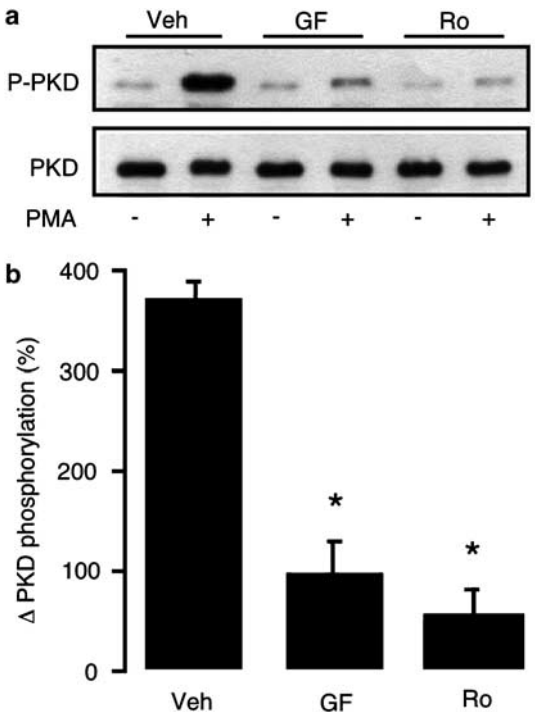

Figure 8 Effects of GF019203X and Ro31-8220 on the phosphorylation status of PKD following exposure to PMA. ARVM were treated with vehicle (Veh), 1 μM GF109203X (GF) or 1 μM Ro31-8220 (Ro) for 15 min, prior to a 5-min exposure to vehicle or 30 nM PMA, lysis in SDS–PAGE sample buffer and subsequent Western immunoblot analysis. (a) Representative Western immunoblots showing the expression of phosphorylated and total PKD. (b) Quantitative data illustrating the PMA-induced increase in the phosphorylation status of PKD. $^*P<0.05$ *versus* Veh ($n=3$).

with similar relative selectivity. However, Ro31-8220 demonstrated greater potency than GF109203X against all three $p90^{RSK}$ isoforms. Previous data have demonstrated that both GF109203X and Ro31-8220 inhibit mixed PKC isoforms and RSK2 with near equipotency (reported IC_{50} values for inhibition of mixed PKCs and RSK2 were 30 and 50 nM, respectively, for GF109203X and 5 and 3 nM, respectively, for Ro31-8220 (Alessi, 1997)). The present data demonstrate that GF109203X exhibits approximately 30-fold selectivity for PKCα and PKCε when compared to RSK2, with IC_{50} values for PKC isoform inhibition (8–12 nM) close to that described in the original study describing this inhibitor (14 nM) (Toullec *et al*., 1991). The selectivity of GF109203X for PKC isoforms was retained at an elevated ATP concentration of 5 mM, which is akin to that estimated in ventricular myocytes (Allue *et al*., 1996). Ro31-8220 exhibited considerably lower (approximately six-fold) selectivity for PKC isoforms over RSK2. Interestingly, this difference in the relative selectivity of the two inhibitors arose from a less potent inhibition of RSK2 by GF109203X when compared to Ro31-8220, since both bisindolylmaleimides inhibited PKC isoforms with comparable potency.

Previous evidence that bisindolylmaleimide PKC inhibitors have nonspecific inhibitory effects on the activity of a number of kinases has relied on *in vitro* kinase assays (Alessi, 1997; Davies *et al*., 2000). More recently, a proteomic screen has identified a number of cellular targets for bisindolylmaleimides (Brehmer *et al*., 2004), consistent with potential nonspecific effects also in the intact cell. Nevertheless, our previous work has drawn into question the validity of extrapolating data from *in vitro* kinase assays to the intact cellular environment, by demonstrating that GF109203X and Ro31-8220 do not inhibit $p70^{S6K}$ in the intact ARVM (Roberts *et al*., 2004). Unlike the contrasting effects of the bisindolylmaleimide inhibitors on $p70^{S6K}$ activity *in vitro versus* in the intact ARVM, their potent inhibition of $p90^{RSK}$ activity *in vitro* was reflected by their inhibition of such activity also in the cellular environment. Thus, both GF109203X and Ro31-8220 showed significant inhibition of $p90^{RSK}$ activity in intact ARVM, at concentrations $\geqslant 3$ and $\geqslant 1\,\mu$M, respectively. These data suggest that $p90^{RSK}$ inhibition may contribute to the functional effects of bisindolylmaleimides in ARVM and potentially in other cell types. Importantly, nonspecific inhibition of cellular $p90^{RSK}$ activity by the two PKC inhibitors may require re-evaluation of some data from previous studies that used these agents as pharmacological tools, particularly to investigate the roles of PKC isoforms in (patho)physiological processes where $p90^{RSK}$ activity is likely, or indeed known, to play a key regulatory role. Of particular interest is the use of these inhibitors to implicate PKC isoforms in the stimulation of NHE1 activity by a variety of stimuli in ARVM (Yasutake *et al*., 1996; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000; Snabaitis *et al*., 2002).

Experiments that have used GF109203X and inhibitors of the ERK/$p90^{RSK}$ pathway (e.g. PD98059 and U0126) to study the signalling mechanisms through which thrombin (Yasutake *et al*., 1996), angiotensin II (AT_1) (Gunasegaram *et al*., 1999) and α_{1A}-adrenergic (Snabaitis *et al*., 2000) receptors stimulate NHE1 activity in ARVM have led to the conclusion that PKC and $p90^{RSK}$ participate in two parallel and necessary pathways that facilitate such a response (Avkiran & Haworth, 2003). However, in the light of the present data, it is possible that the common inhibitory effects of GF109203X and PD98059/U0126 could have arisen from the inhibition of a contiguous signalling pathway at proximal and distal points, by targeting ERK and therefore $p90^{RSK}$ activation (PD98059/U0126) or $p90^{RSK}$ activity (GF109203X). Notably, previous studies in our laboratory that have used GF109203X as a PKC inhibitor have employed concentrations $\leqslant 1\,\mu$M (Yasutake *et al*., 1996; Gunasegaram *et al*., 1999; Snabaitis *et al*., 2000; Snabaitis *et al*., 2002), which did not inhibit cellular $p90^{RSK}$ activity significantly in the present study (Figure 6). Thus, nonspecific inhibition of $p90^{RSK}$ is unlikely to have been primarily responsible for the inhibitory effects of GF109203X on the stimulation of NHE1 activity by the pertinent GPCR agonists in intact ARVM. In addition, other studies in noncardiac cell types have used alternative tools to manipulate cellular PKC activity, and have supported a role for PKC and/or specific PKC isoforms in NHE1 regulation (Chen & Wu, 1995; Maly *et al*., 2002). Nevertheless, further investigation, using complementary techniques, is required to confirm the involvement of PKC isoforms in the regulation of NHE1 activity in ARVM, particularly since, unlike $p90^{RSK}$ (Takahashi *et al*., 1999), PKC isoforms do not appear to directly phosphorylate the regulatory C-terminal domain of NHE1 (Fliegel *et al*., 1992).

The discrepancy between the lack of inhibition of $p70^{S6K}$ (Roberts *et al*., 2004) and the significant inhibition of $p90^{RSK}$

(present study) by GF109203X and Ro31-8220 in intact ARVM, despite the potent inhibition of both kinases by the two compounds *in vitro* (Alessi, 1997; Davies *et al*., 2000), can potentially be explained through differences in the intracellular localisation and/or regulation of the pertinent proteins. Bisindolylmaleimide inhibitors exert their actions through competitive inhibition at the ATP-binding site within the catalytic domains of PKC isoforms (Toullec *et al*., 1991), and it is assumed that GF109203X and Ro31-8220 act in a similar manner to inhibit the catalytic activity of $p70^{S6K}$ and $p90^{RSK}$ (Alessi, 1997). It is possible that there is variable access of bisindolylmaleimides to the catalytic domains of the two kinases in the intact ARVM, due to differences in their localisation and/or regulation. Although both kinases are predominantly cytosolic in localisation under basal conditions in other cell types (Pullen & Thomas, 1997; Frödin & Gammeltoft, 1999), there are currently no data describing the cellular localisation of either $p70^{S6K}$ or $p90^{RSK}$ in ARVM. Furthermore, it is possible that the kinases occupy distinct subcellular compartments in the activated state, allowing differential access to pharmacological inhibitors. Indeed, activated $p70^{S6K}$ has been shown to exist in a large multi-protein complex (Hannan *et al*., 2003), which may restrict inhibitor access to the kinase. Such differences could lead to greater access and preferential binding of GF109203X and Ro31-8220 to $p90^{RSK}$ compared with $p70^{S6K}$, resulting in more potent inhibition of the former in the intact cell.

eEF2 is a cytoplasmic protein that catalyses the movement of the ribosome along mRNA during translation (which is essential for the extension of the polypeptide chain) and is regulated through phosphorylation by eEF2K (Ryazanov *et al*., 1997). eEF2K itself is negatively regulated by the upstream kinases $p90^{RSK}$ and $p70^{S6K}$ through phosphorylation at S366 (Wang *et al*., 2001b), such that activation of either pathway results in the inhibition of eEF2K activity and the consequent attenuation of eEF2 phosphorylation (Wang *et al*., 2001b). Interestingly, in the present study, the attenuation of eEF2 phosphorylation following heterologous expression of caMEK1 was not inhibited by GF109203X or Ro31-8220, despite the significant attenuation of eEF2K phosphorylation by both agents. In contrast, the inhibition of ERK activation by U0126 abolished both the increase in eEF2K phosphorylation and the decrease in eEF2 phosphorylation that arose from the heterologous expression of caMEK1, as previously shown (Wang & Proud, 2002). The discrepant effects of bisindolylmaleimides and the consistent effects of U0126 on the phosphorylation status of eEF2K *versus* eEF2 may indicate the existence of MEK/ERK-dependent but $p90^{RSK}$-independent mechanisms that regulate eEF2 phosphorylation in ARVM. Thus, in addition to the pharmacological characterisation of GF109203X and Ro31-8220, our study provides novel mechanistic information, which is of potential relevance to the regulation of protein synthesis in ARVM and thus warrants further investigation.

In conclusion, our data show that GF109203X and Ro31-8220 are potent inhibitors of $p90^{RSK}$ activity *in vitro* and target all three $p90^{RSK}$ isoforms tested (RSK1, RSK2 and RSK3). Our data also show that both GF109203X and Ro31-8220 significantly inhibit $p90^{RSK}$ activity in the intact ARVM, at concentrations $\geqslant 3$ and $\geqslant 1\,\mu$M, respectively. Although GF109203X appears to be more selective than Ro31-8220 as a PKC inhibitor, both *in vitro* and in intact ARVM, care must still be taken in selecting an appropriate concentration of either GF109203X or Ro31-8220 and in interpreting data arising from the use of these agents in cellular systems such as ARVM, due to their potential to inhibit $p90^{RSK}$ as well as PKC activity.

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