The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1β (Rsk-2) and p70 S6 kinase

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Abstract The protein kinase C (PKC) inhibitors Ro 318220 and GF 109203X have been used in over 350 published studies to investigate the physiological roles of PKC. Here we demonstrate that these inhibitors are not selective for PKC isoforms as was previously assumed. Ro 318220 inhibited MAPKAP kinase-1β (also known as Rsk-2) in vitro (IC_{50} 3 nM) more potently than it inhibited mixed PKC isoforms (IC_{50} 5 nM), and it also inhibited p70 S6 kinase (IC_{50} 15 nM). GF 109203X also potently inhibited MAPKAP kinase-1β (IC_{50} 50 nM) and p70 S6 kinase (IC_{50} 100 nM) with similar potency to PKC isoforms (IC_{50} 30 nM). The inhibition of MAPKAP kinase-1β, p70 S6 kinase, and probably other protein kinases, may explain many of the effects previously attributed to PKC.

Key words: Protein kinase inhibitor; PKC; p70 S6 kinase; Rsk

1. Introduction

The α, β and γ isoforms of protein kinase C (PKC, hereafter termed 'mixed PKC isoforms') are activated by the second messenger diacylglycerol, which is generated in response to a variety of agonists [1]. The mixed PKC isoforms are thought to mediate numerous signal transduction processes including the control of cell growth, differentiation and homeostasis. Much of the evidence implicating PKC in these events has been based on the use of either tumour-promoting phorbol esters, which are thought to activate PKC by mimicking diacylglycerol, and on the use of two small cell permeable inhibitors of PKC namely Ro 318220 [2] and GF 109203X [3]. These two compounds are bisindoylmaleimides which differ from each other in two functional groups and are analogues of staurosporine. They are both potent inhibitors of the α, β and γ isoforms, with IC_{50} values in the nM range, and compete for the ATP-binding site on PKC [3–5]. They have been reported to be specific inhibitors in that two other serine/threonine-specific protein kinases, protein kinase A (PKA) and a calcium/calmodulin-dependent protein kinase(s), and several receptor protein tyrosine kinases were only inhibited at 1000-fold higher concentrations [2,3]. Since the introduction of Ro 318220 and GF 109203X, these compounds have been used in over 350 published studies to implicate mixed PKC isoforms in the regulation of many cellular processes. However, the validity of these findings rests on the assumption that they inhibit mixed PKC isoforms selectively when used in cell-based assays at low micromolar concentrations. Here we show that two other protein kinases, MAP kinase-activated protein (MAPKAP) kinase-1β (also known as Rsk-2) and p70 S6 kinase, which become activated in response to a variety of extracellular signals including phorbol esters, are inhibited by Ro 318220 and GF 109203X at concentrations similar to those which inhibit mixed PKC isoforms. These studies indicate that neither inhibitor is sufficiently specific to implicate PKC in the regulation of a physiological process, and that many of the effects ascribed to PKC may instead result from the inhibition of MAPKAP-K1β, p70 S6 kinase or other protein kinases.

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Abbreviations: PKB, protein kinase B; MAP kinase, mitogen-activated protein kinase; MAPKK-1, MAP kinase kinase-1; MAPKAP-kinase, MAP kinase-activated protein kinase; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C

2. Materials

Ro 318220 was a kind gift from Dr. D. Bradshaw (Roche Pharmaceutical Company, Welwyn Garden City, UK) and GF 109203X was purchased from Calbiochem (Nottingham, UK). Both inhibitors were dissolved in DMSO at a concentration of 50 mM and stored in aliquots at −20°C. Mixed PKC isoforms and histone H1, were purchased from Boehringer Mannheim (Lewis, UK) and myelin basic protein (MBP) was from Life Technologies (Paisley, UK). MAPKAP-K1β [6] and MAPKAP-K2 [7] were purified from rabbit skeletal muscle by Dr. N. Morrice (MRC Protein Phosphorylation Unit, Dundee). The p70 S6 kinase was partially purified from the livers of rats treated with cycloheximide [8] by Dr. S. Dale (MRC Protein Phosphorylation Unit, Dundee). Protein kinase Bα (also known as c-Akt or RAC kinase) was immunoprecipitated from IGF-1 stimulated 293 cells overexpressing this kinase [9]. c-Raf1 was obtained from Sf9 cells infected with bacculoviruses encoding c-Raf1, Ras and lck [10]. MAP kinase kinase-1 (MAPKK-1) and p42 MAP kinase were expressed in *E. coli* as glutathione S-transferase fusion proteins [11] and then activated in vitro with c-Raf and activated MAPKK-1, respectively [12]. All peptides used to assay protein kinases, and TTYADFIASGRTGRRNAIHD (the specific peptide inhibitor of cyclic AMP dependent protein kinase-PKI) were synthesised by Mr. F.B. Caudwell at Dundee on an Applied Biosystems 431A peptide syntheser. Their purity was (>95%) and their concentrations were determined by quantitative amino acid analysis.

2.1. Protein kinase assays

All protein kinases except c-Raf and MAPKK-1 were assayed as follows: a 40 μl assay mix was prepared containing protein kinase (0.2 U/ml) in 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 2.5 μM PKI, protein kinase substrate (30 μM), and the indicated concentration of Ro 318220 or GF 109203X. After incubation on ice for 10 min the reaction was started by the addition of 10 μl of 50 mM magnesium acetate and 0.5 mM [γ^{32}P]ATP (100–200 cpm/pmol). For the assay of mixed isoforms of PKC, 20 μM diacylglycerol, 0.5 mM $CaCl_2$, and 100 μM phosphatidylserine were also present in the incubations. The assays were carried out for 15 min at 30°C, then terminated and analysed as described [11]. One unit of activity was that amount of enzyme that catalysed the phosphorylation of 1 nmol of substrate in 1 min. The final concentration of DMSO in each assay was 1% (by vol). This concentration of DMSO does not inhibit any of these enzymes. Mixed isoforms of PKC were assayed using histone H1 as substrate, while MAPKAP-K1β and p70 S6 kinase were assayed using the peptide KKRNRTL-

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SVA [8]. PKB was assayed with the peptide GRPRTSSFAEG [9] and MAPKAP-K2 was assayed with the peptide KKLNRTLSVA [13]. p42 MAP kinase was assayed using MBP, and MAPKK-1, and c-Raf1 were assayed as described in [11].

3. Results

3.1. Effect of Ro 318220 and GF 109203X on protein kinases activated by growth factors, cytokines and cellular stresses

As reported previously [2], the mixed isoforms of PKC were potently inhibited by Ro 318220, with an IC_{50} of 5 nM in our assay (Fig. 1A). In contrast, a number of protein kinases activated by growth factors (c-Raf1, MAPKK-1, p42 MAP kinase) and one protein kinase that is activated by cellular stresses and proinflammatory cytokines (MAPKAP-K2) were not inhibited significantly by Ro 318022 in vitro (Fig. 1A). PKBα, an enzyme that is activated in response to insulin and growth factors, was inhibited by Ro 318220 (IC_{50} of 1 μM, Fig. 1B) similar to the IC_{50} for PKA. However, to our surprise, MAPKAP-K1β an enzyme which lies immediately downstream of p42 and p44 MAP kinases and which is activated in response to every agonist that stimulates this pathway, was inhibited by Ro 318220 even more potently than the mixed PKC isoforms (IC_{50} = 3 nM, Fig. 1B). The p70 S6 kinase, which lies on a distinct growth factor-stimulated signalling pathway from MAPKAP-K1β, was also potently inhibited by Ro 318220 (IC_{50} = 15 nM, Fig. 1B).

Similar results were obtained using GF 109203X instead of Ro 318220. As reported previously [3], GF 109203X inhibited the mixed isoforms of PKC (IC_{50} = 30 nM) without inhibiting PKBα (Fig. 2) or c-Raf, MAPKK-1 and p42 MAP kinase (data not shown). However MAPKAP-K1β and p70 S6 kinase were potently inhibited by this compound with IC_{50} values of 50 nM and 100 nM, respectively (Fig. 2).

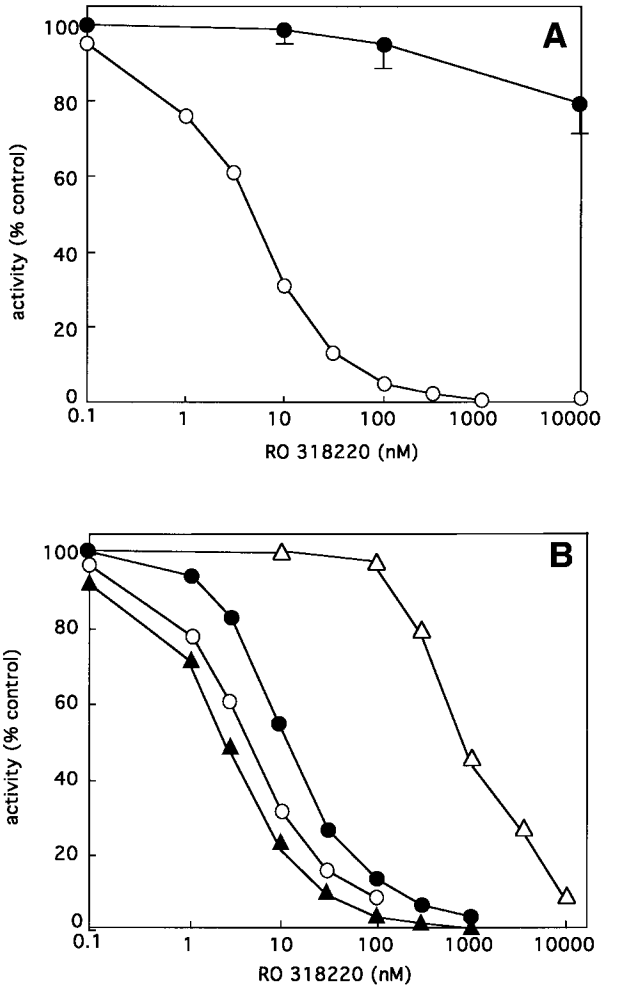

Fig. 1. Effect of Ro 318022 on the activity of a variety of agonist regulated protein kinases. A: Effect of Ro 318220 on mixed PKC isoforms (○) and on c-Raf, MAPKK-1, p42 MAP kinase, and MAPKAP-K2 (●). These 4 kinases were all affected similarly by Ro 318220. B: Effect of Ro 318022 on mixed PKC isoforms (○), MAPKAP-K1β (▲), p70 S6 kinase (●) and PKB (△). The results are presented relative to control incubations in which the inhibitor was omitted and are shown as the average of 3 experiments with each determination carried out in triplicate. The error for each data point is < 5% unless otherwise indicated.

4. Discussion

In this communication we have shown that Ro 318220 and GF 109203X are not selective inhibitors of the mixed PKC isoforms. We demonstrated that both MAPKAP-K1β and p70 S6 kinase are inhibited with similar potency by Ro 318220 and GF 109203X (Figs. 1 and 2). The amino acid sequence of the catalytic domain of p70 S6 kinase and the N-terminal kinase domain of MAPKAP-K1β are both 40% identical to the catalytic domain of PKCα. This degree of identity is similar to that between the catalytic domains of PKCα and PKA or between PKCα and PKBα; PKA and PKBα are inhibited by Ro 318220 at 100–1000-fold higher concentration than mixed PKC isoforms. These findings emphasise the danger in assuming that any given kinase inhibitor is specific, unless it has been tested against a substantial number of other kinases, and not simply those kinases which are most closely related in structure.

MAPKAP-K1β and p70 S6 kinase both lie on distinct signalling pathways that are activated in response to growth factors and signals that also activate mixed PKC isoforms. Thus the blockade of a growth-factor mediated effect by Ro 318220 or GF 109203X may be explained by inhibition of MAPKAP-K1β and/or p70 S6 kinase, even in situations where the effect of the growth factor is mimicked by phorbol esters.

PKC is known to be stimulated during antigen-induced T-cell activation and Ro 318220, GF 109203X and similar bisindoylmaleimide derivatives have been shown to suppress antigen-driven T-cell proliferation in cultured cells and in vivo, raising interest in the potential use of PKC inhibitors as immunosuppressants [5,14]. However, since the immunosuppressant drug rapamycin is a potent and specific inhibitor of the activation of p70 S6 kinase [15], it is possible that the immunosuppressant effects of Ro 318220 and GF 109203X result from inhibition of p70 S6 kinase rather than PKC. The therapeutic use of drugs related to Ro 318220 and GF 109203X will also need be evaluated very carefully in the light of the recent discovery of the cause of Coffin-Lowry syndrome [16]. This genetic disease, which leads to progressive skeletal deformation and severe mental retardation results from the mutational-inactivation of the same MAPKAP-K1 isoform (MAPKAP-K1β) used in this study [16].

It will also be important for investigators who have examined the effects of Ro 318220 and GF 109203X on any cellu-

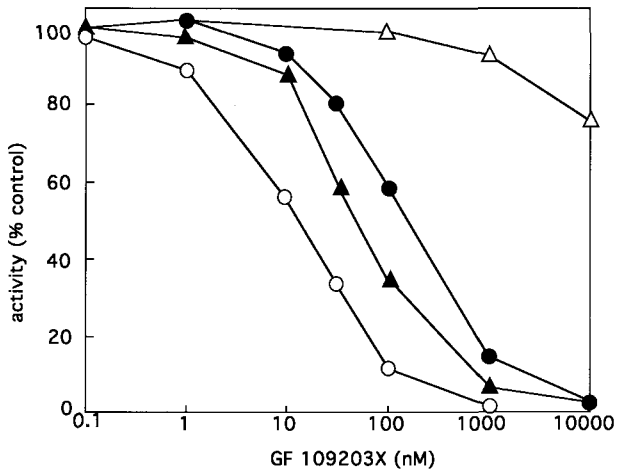

Fig. 2. Effect of GF 102203X on the activity variety of agonist regulated protein kinases. Effect of GF 109203X on mixed PKC isoforms (○), MAPKAP-K1β (▲), p70 S6 kinase (●), and PKBα (△). The results are presented relative to control incubations in which the inhibitor was omitted and are shown as the average of 2 experiments with each determination carried out in triplicate. The error for each data point is < 5% SEM.

lar process to check whether that process is also blocked by rapamycin [15] or by PD 98059 [17]. The last mentioned drug, which is an extremely specific inhibitor of the activation of MAPKK-1, prevents the activation of p42 and p44 MAP kinases and hence the activation of MAPKAP-K1 [17].

Evidence that Ro 318220 may not be a specific inhibitor of mixed PKC isoforms in cell based assays was recently reported by others [18]. In these studies Ro 318220 prevented the induction of MAP kinase phosphatase-1 and the transcription factor c-Fos by growth factors, and stimulated activation of the stress-activated protein kinase JNK in the absence of growth factors, even after depletion of mixed PKC isoforms by prolonged treatment with phorbol esters. The suppression of c-Fos induction by Ro 318220 may result from the inhibition of MAPKAP-K1β since this enzyme has been implicated in the nerve growth factor-stimulated induction of c-Fos in PC12 cells [19]. Inhibition of MAPKAP-K1β might also underlie the suppression of MAP kinase phosphatase-1 induction by growth factors, since PD 98059 prevents the induction of this phosphatase by IGF-1 in L6 myotubes (T. Lewis, S. Keyse, and D. Alessi, unpublished work). Beltman et al. [18] reported that GF 109203X, when used at a concentration of 2.5 μM, did not mimic the effects of Ro 318220 used at a concentration of 5 μM. This is also consistent with the possible involvement of MAPKAP-K1β which is inhibited by 15-fold less potently by GF 109203X than by Ro 318220 (Figs. 1 and 2).

In summary, Ro 318220 potently inhibits at least three types of protein kinase (and probably more). To my knowledge the only three inhibitors of serine/threonine-specific protein kinases which have so far survived rigorous studies of their specificity, both in vitro and in cell-based systems, are the p38/RK MAP kinase inhibitor SB 203580 [20], the inhibitor of MAPKK-1 activation PD 98059 [17], and rapamycin [15].

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