Effects of the selective protein kinase C inhibitor Ro 31–7549 on human angiotensin II receptor desensitisation and intracellular calcium release

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Abstract The mechanism underlying type I angiotensin II (Ang II) receptor (AT1 receptor) desensitisation is unknown. Structural features suggest it may be a substrate for protein kinase C (PKC). The effects of a selective PKC inhibitor, Ro 31–7549, on receptor desensitisation were investigated in CHO cells expressing the human AT1 receptor. Desensitisation was demonstrated with respect to the calcium response to Ang II in Fura-2 -loaded cells. Ro 31–7549 had no effect on desensitisation. However, pretreatment with Ro 31–7549 caused a dose-dependent reduction in calcium release from intracellular stores. PKC may therefore act at a locus distal from the receptor itself.

Key words: Angiotensin receptor; Desensitisation; Protein kinase C; Calcium release; Fura 2; CHO cell

1. Introduction

Angiotensin II (Ang II) plays a crucial role in controlling blood pressure through its potent vasoconstrictor effect, and in mineral homeostasis by effects on adrenal aldosterone synthesis [1]. These effects are mediated by the AT1 subtype of the Ang II receptor (AT1 receptor) via coupling to a guanine nucleotide regulatory protein (G protein) [2] and leading to activation of phospholipase C (PLC) - beta. This, in turn, results in the hydrolysis of phosphatidyl inositol, with the consequent production of the second messengers inositol (1,4,5) trisphosphate (InsP3) and *sn*-1,2-diacylglycerol (DAG) [3]. InsP3 acts through the InsP3 receptor to release calcium from intracellular $InsP_3$-sensitive calcium stores [4–5] while DAG acts within the plane of the membrane to activate protein kinase C (PKC). A process of calcium entry into the cell also occurs through calcium channels located on the plasma membrane [6]. In addition, following initial stimulation by Ang II, the AT1 receptor undergoes a process of desensitisation [7]. The mechanism underlying this process has yet to be defined for this receptor.

Four putative sites for phosphorylation by protein kinase C (PKC) have been identified in the carboxy terminal of the human AT1 (hAT1) receptor [8]. This suggests that PKC has a direct effect on the receptor itself and thus may be important in AT1 receptor-mediated signal transduction or, by analogy to the effect of cAMP-dependent protein kinase (PKA) in the β-adrenergic receptor system [9], in receptor desensitisation. However, a specific role for PKC in AT1 receptor function is yet to be established. Activation of PKC inhibits Ang II dependent PLC activation in vascular smooth muscle cells [10], while studies in isolated perfused rat kidney, using the non-selective PKC inhibitor staurosporine, have suggested that PKC potentiates Ang II-induced vasoconstriction [11]. In contrast, in bovine adrenal glomerulosa cells inhibition of PKC partially attenuates Ang II induction of c-fos mRNA [12] and aldosterone secretion in bovine [13] and rat [14] adrenal glomerulosa cells, respectively. In terms of Ang II receptor desensitisation, use of the non-selective PKC inhibitor, staurosporine, or activation of PKC using phorbol ester has yielded different results in different systems. Shimuta et al. [15], have demonstrated that Ang II-induced desensitisation in guinea pig ileum and cultured guinea pig myocytes is inhibited by staurosporine and enhanced by phorbol ester. A similar effect of staurosporine has been shown for Ang II receptors coupled to chloride channels in *Xenopus* oocytes [16]. However, desensitisation was shown to be independent of PKC in neonatal cardiomyocytes [17].

In this paper we report studies on chinese hamster ovary (CHO) cells stably transfected with the human AT1 receptor gene (CHO.hAT1 cells), in which we have investigated the role of PKC in AT1 receptor function using the selective PKC inhibitor, Ro 31–7549 [18–22].

2. Materials and Methods

2.1. Fura-2 studies

CHO cells were routinely cultured in Ham's F12 medium containing 10% v/v FBS (complete medium). After harvesting cells using trypsin-EDTA (0.5% w/v trypsin; 0.02% w/v EDTA), cells were allowed to re-equilibrate in complete medium, in the presence or absence of the various inhibitors and activators of PKC described below, for 30 min prior to washing twice into 5 mM HEPES buffer containing 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 11 mM D-glucose and 0.1% bovine serum albumin (BSA) (w/v), pH 7.4. Cells were loaded in fresh HEPES buffer (containing BSA) at 37°C for 30 min with 4 μM Fura-2/AM (Calbiochem-Novabiochem, UK), and washed twice in HEPES buffer without BSA and resuspended in a final volume of 2 ml. For calcium replete conditions, extracellular calcium was increased to 1mM with $CaCl_2$. Measurements of intracellular calcium were conducted in an RF-5000 dual wavelength fluorescence spectrophotometer (Shimadzu) at wavelengths of 340 nm and 380 nm, at a temperature of 25°C. The cell suspensions were calibrated for leakage using manganese chloride (200 μM final concentration) followed by diethylenetriaminepentaacetic acid solution treated with calcium carbonate (CaDTPA) (400 μM). Estimates for Fura-2 fluorescence at infinite and zero calcium concentrations were obtained using 100 μM digitonin and 50 mM EGTA (final concentrations), respectively. Values for intracellular calcium were calculated by the formula used in [23], assuming a dissociation constant of Fura-2 for calcium at 25°C of 150 nM [24].

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Abbreviations: Ang II, angiotensin II; AT1 receptor, type I angiotensin II receptor; hAT1 receptor, human AT1 receptor; PKC, protein kinase C; G protein, guanine nucleotide regulatory protein; PLC, phospholipase C; InsP3, inositol (1,4,5) trisphosphate; DAG, *sn*-1,2-diacylglycerol; CHO, chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol.

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2.2. Ligand binding assays

Cells were prepared as above but, instead of loading with Fura-2/AM, cells were aliquoted into eppendorf tubes at a density of approximately 10^6 cells/tube (in 50 μl HEPES buffer containing 0.1% BSA). 50 μl of buffer (uncompeted tubes) or buffer containing unlabelled Ang II (final concentration in 150 μl final volume ranging from 0.1 nM – 100 nM) was added, followed by 50 μl [^{125}I]^{1}Sar,^{8}Ile-Ang II (2000 Ci/mmol; Amersham; final concentration 0.15 nM). After incubation for 1 h at 22°C, tubes were transferred to an ice-bath and 850 μl ice-cold assay buffer was added. The suspension was then centrifuged at 6000 × *g* for 20 s at 4°C. Supernatants were aspirated and a further aliquot of ice-cold buffer added. After a second wash, cells were pelleted at 12,000 × *g* and counted by gamma counter. Values for receptor capacity and affinity were obtained by Scatchard analysis.

3. Results

3.1. Homologous desensitisation of AT1 receptor

The CHO·hAT1 cells expressed high affinity binding for Ang II which was displaceable by Dup 753 (data not shown). To examine receptor desensitisation in these cells we determined the effect of serial exposure to Ang II on calcium mobilisation. A sub-maximal dose of Ang II (20 nM) induced a rapid and transient peak of calcium mobilisation (179 ± 21 nM above basal; $n = 6$) followed by a sustained mobilisation of calcium (Fig. 1). No change in basal calcium was observed for CHO · NEO cells (stably transfected with only the neomycin resistance gene) (data not shown). 100 nM Ang II was found to produce a maximal stimulation under these conditions (270 ± 20 nM calcium peak increase above basal; $n = 3$). Following initial exposure to 20 nM Ang II, the sustained plateau phase of calcium mobilisation was allowed to proceed for periods of up to 20 min after which a second maximal dose of Ang II (100 nM) was added. As can be seen in Fig. 1 no further peaks of calcium mobilisation were observed in response to Ang II. However, subsequent addition of 0.1% FBS triggered a calcium response, indicating that the calcium stores were not depleted and suggesting that serum-initiated calcium release is not similarly desensitised.

3.2. Effects of Ro 31–7549

Pre-treatment of CHO.hAT1 cells with 15 μM Ro 31–7549 had no effect on AT1 receptor desensitisation (data not shown). Pre-treatment (30 min) with either the non-selective PKC inhibitor, staurosporine (100 nM), or PKC activators, phorbol 12-myristate 13-acetate (PMA; 1 μM) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; 10 μM), was also without effect (data not shown). However, pre-treatment with Ro 31–7549 produced a small, but significant, reduction in the height of the calcium

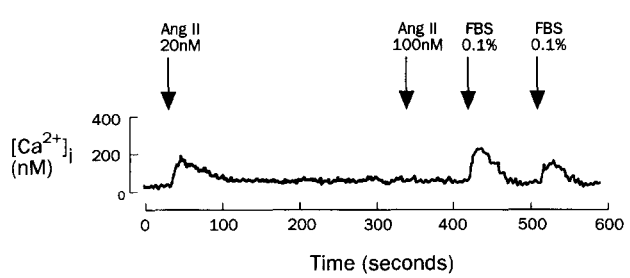

Fig. 1. Calcium mobilisation in CHO cells expressing the human AT1 receptor in response to sub-maximal Ang II (20 nM), showing clear desensitisation towards subsequent maximal Ang II (100 nM) stimulation.

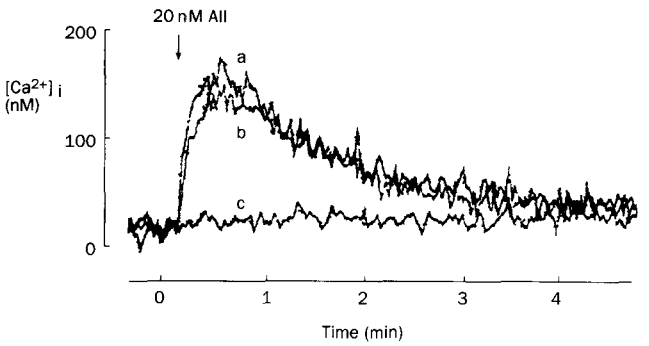

Fig. 2. Representative traces showing change in intracellular calcium in reponse to Ang II (20 nM) in (a) CHO·hAT1 cells pretreated with DMSO vehicle; (b) CHO·hAT1 cells pretreated with Ro 31–7549 (15 μM); and (c) CHO·NEO cells.

peak, reducing the percentage increase in peak calcium over basal by 27 ± 8% ($n = 3$) relative to cells treated with DMSO vehicle alone (Fig. 2). Pre-treatment with the CaM kinase inhibitor KN-62 (Research Biochemicals International) (100 nM) had no such effect (data not shown). After removal of extracellular calcium using EGTA (2 mM final concentration), the effect of 15 μM Ro 31–7549 was much more pronounced, reducing the calcium peak by 66 ± 5% ($n = 4$) compared to DMSO treated control cells (Fig. 3). This inhibitory effect was dose dependent and was also observed in CHO · hAT1 cells pre-treated with staurosporine (Fig. 3). Despite almost complete inhibition of calcium release from intracellular stores, high doses of Ro 31–7549 (22.5 μM) had no affect on receptor desensitisation (data not shown). Furthermore, pre-treatment with Ro 31–7549 (22.5 μM) had no significant effect on either AT1 receptor concentration or affinity in these cells (Table 1).

4. Discussion

Previous work by other groups has made use of the transfected CHO cell system to study rat AT1a receptor function [25–28]. However, this is the first report of functional analysis of the human AT1 receptor in this system. The hAT1 receptor showed profound homologous desensitisation (Fig. 1), a phenomenon which was unaffected by modulation of PKC by inhibitors (Ro 31–7549 and staurosporine), or activators (PMA and OAG), or the CaM kinase inhibitor, KN-62. This suggests that PKC has no effect on hAT1 receptor desensitisation in this system. This lack of effect has also been shown in neonatal cardiomyocytes [17] but contrasts with the effects of PKC on Ang II receptor desensitisation in guinea pig tissue [15] and on the *Xenopus* angiotensin receptor (xAT) [16]. The differences between these observations may be the result of species differences, either in Ang II receptor structure, or in the nature of target cell specific signal transduction and desensitisation mechanisms. It is possible that receptor internalisation may play a part in AT1 receptor desensitisation. It has been shown that AT1 receptor internalisation occurs in bovine [29] and rat [30] adrenal glomerulosa cells and that rat AT1a receptors expressed in CHO cells also undergo internalisation [28]. It is notable, however, that in neonatal cardiomyocytes [17], where PKC had no effect on desensitisation, there was also no loss of surface receptor capacity associated with desensitisation.

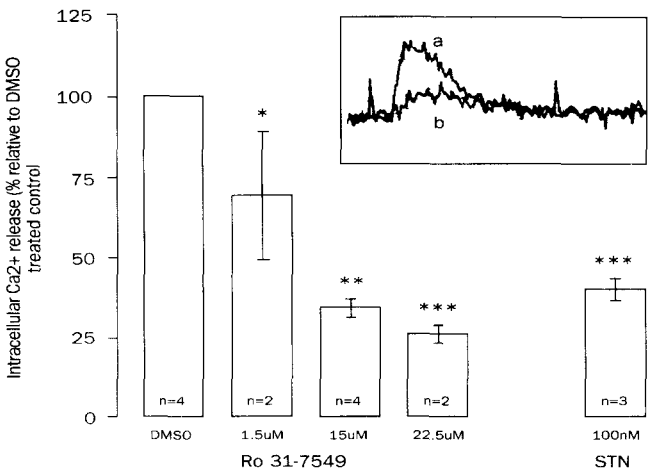

Fig. 3. Dose-dependent reduction in calcium peak after pretreatment with Ro 31 7549 under calcium deplete conditions. $*P<0.05$; $**P<0.01$; $***P<0.001$, (compared to DMSO control) Student's t-test. *INSET*: Representative trace showing effect of 15 μM Ro 31–7549 (a), compared with DMSO-treated (control) cells (b), under calcium-deplete conditions.

Despite the apparent lack of PKC effect on desensitisation it is clear that PKC can affect Ang II induced calcium mobilisation in CHO.hAT1 cells. Inhibition of PKC by Ro 31–7549 resulted in a substantial decrease in calcium release from intracellular stores (Fig. 3). This suggests that PKC in some way facilitates Ang II-stimulated calcium release. However, this does not appear to be at the level of the AT1 receptor itself, since Ro 31–7549 had no effect on binding of labelled Ang II (Table 1). Inhibition of PKC activation of PLC-beta, and thus a reduction in InsP3 formation, could explain the results seen in Fig. 3, however, PKC phosphorylation of PLC-beta has been shown to be inhibitory to this moiety [32]. One possible point at which PKC may act could be at the level of the InsP3 receptor. As PKC has been shown to phosphorylate the InsP3 receptor [31], it is tempting to propose a role for PKC in sensitising the latter.

In our study, the inhibition of PKC by Ro 31–7549 appeared to induce calcium influx to an extent which virtually masked the attenuation in calcium release identified under calcium deplete conditions (Fig. 2b). This was greater than the influx normally expected concomitantly with calcium release from intracellular stores. It has been shown that, at doses above 10nM Ang II, calcium influx during the initial transient calcium peak is attenuated, or that the rate of influx is no greater than the basal rate [33,34]. However, a study of rat AT1 receptor expressed in CHO cells suggested there was a contribution from calcium influx during the initial transient peak [25]. Recent data has indicated that Ang II-induced activation of PKC is dependent on calcium entry [35]. It is possible, therefore, that inhibition of PKC prevents some feedback suppression of calcium entry by direct interaction of PKC with a calcium channel.

Table 1
Angiotensin II binding characteristics in CHO·hAT1 cells

	K_d (nM)	B_{max} (fmol/mg protein)
Control (DMSO)	0.74 ± 0.07	389 ± 35
Ro 31-7549 (22.5 μM)	0.84 ± 0.09	369 ± 41

Values represent mean ± S.D. from three determinations.

In conclusion, we have demonstrated a lack of effect of PKC on desensitisation of the human AT1 receptor expressed in CHO cells. However, inhibition of PKC by Ro 31–7549 reduces release of calcium from intracellular stores and leads to an apparent compensatory influx of calcium. One could speculate that sensitisation of the InsP3 receptor may be a novel role for PKC in Ang II stimulated signal transduction and that PKC may also be important in controlling calcium channel opening.

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