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Protein kinase C rapidly up-regulates the number of AT_1 angiotensin receptors on cultured rat intestinal epithelial (RIE-1) cells

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Abstract

Addition of 12-*O*-tetradecanoylphorbol-13-acetate to RIE-1 rat intestinal epithelial cells stimulated a rapid (mean 3-fold) increase in the subsequent binding of ^{125}I-labelled angiotensin II which was reversed or prevented when cellular protein kinase C was depleted. The increased binding was due, in part, to an up-regulation in the number of AT_1 angiotensin receptors on RIE-1 cells, without any significant change in their binding affinity. Since this rapid up-regulation was independent of receptor synthesis, it may result from an increased availability (to extracellular ligand) of preformed, but previously 'cryptic', AT_1 angiotensin receptors.

Key words: Protein kinase C; Up-regulation; AT_1 angiotensin receptor; RIE-1 cell; Intestinal epithelium

1. Introduction

The investigation of cellular growth control has tended to focus on cultured fibroblastic cell lines such as Swiss 3T3 cells which respond mitogenically to activation of various signalling pathways [1,2]. For example, polypeptide growth factors elicit mitogenic responses in these cells by activating receptor tyrosine kinases [3–6], whereas a variety of other mitogenic agonists share the ability to activate receptors that couple (via G proteins) to hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) [7]. However, in contrast to fibroblasts, the agonists and signalling pathways involved in regulating the proliferation of epithelial cells are less well characterised. In order to address this issue, we have investigated factors that regulate proliferation in a cultured epithelial cell line (RIE-1) derived from the rat small intestine [8].

Like Swiss 3T3 cells, RIE-1 cells are stimulated to proliferate through the activation of receptor tyrosine kinases by polypeptide growth factors such as epidermal growth factor (EGF), insulin-like growth factor I (IGF-I) and insulin [9,10]. In order to investigate possible mitogenic signalling via the inositol lipid pathway in RIE-1 cells, we have recently identified the presence of AT_1 angiotensin receptors (which couple to PIP_2 hydrolysis) on these cells [11,12]. Activation of AT_1 receptors by angiotensin II (AII) was sufficient (in the absence of co-administered polypeptide growth factors) to stimulate DNA synthesis and proliferation in RIE-1 cells [12].

In view of their ability to mediate mitogenic signalling, I have begun to characterise the AT_1 receptors expressed by RIE-1 cells. Here, I demonstrate that activation of protein kinase C (PKC) by the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), stimulates a rapid, protein synthesis-independent up-regulation in the number of AT_1 receptors on RIE-1 cells.

2. Materials and methods

TPA and cycloheximide were from Sigma. DuP 753 and PD 123319 were supplied by DuPont and Parke Davis, respectively. AII (Bachem) was iodinated using the soluble lactoperoxidase method [13]. RIE-1 cells were maintained in culture in DMEM/5% newborn calf serum (ICN/Flow) as described [8] and grown to confluence in plastic dishes (Nunc) prior to experimental use.

For [^{125}I]AII binding assays, the cells were treated with test agents at 37°C as described in the individual figure legends. Cells were then washed three times with ice-cold binding medium (BM: PBS supplemented with 0.1 μM KI and 0.1% (w/v) BSA) prior to overnight incubation at 4°C in BM containing [^{125}I]AII (0.5 nM). Non-specific binding was determined in the presence of an excess (10 μM) of unlabelled AII. After three washes with ice-cold BM, the cells were harvested into solubilisation buffer (Triton X-100 1% (v/v), glycerol 10% (v/v), BSA 0.1% (w/v), HEPES 25 mM, pH 7.5) and cell-associated radioactivity was determined using a gamma counter.

For the detection of PKC, cells were lysed directly into hot electrophoresis sample buffer and cellular proteins were subjected to SDS-PAGE under reducing conditions. Following electroblotting onto nitrocellulose, the α and β isoforms of PKC were labelled with a murine monoclonal antibody (MC_5 clone, Amersham). Immune complexes were detected by sequential incubation with a biotinylated sheep anti-mouse second antibody followed by [^{125}I]streptavidin (Amersham).

3. Results

A variety of agents known to regulate the proliferation

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Abbreviations: AII, angiotensin II; BM, binding medium; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphon ic acid; IGF-I, insulin-like growth factor type I; PIP_2; phosphatidylinositol 4,5 bis-phosphate; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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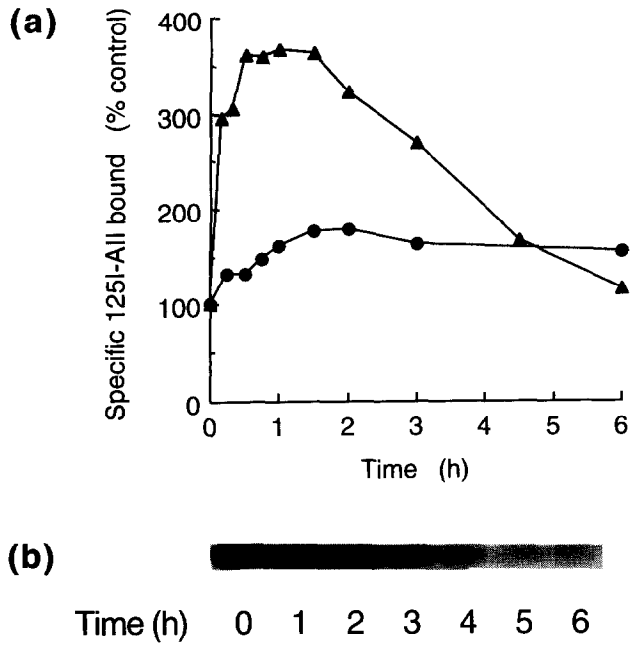

Fig. 1. Time course for the TPA-stimulated increase of [^{125}I]AII binding to RIE-1 cells. (a) Cells were incubated with 3 nM (●) or 300 nM TPA (▲) for the indicated times at 37°C. The specific binding of [^{125}I]AII was then determined. Each point represents the mean of duplicate determinations. (b) Cells were treated with 300 nM TPA at 37°C for the indicated times. PKC was then detected using immunoblotting as described in the text.

of RIE-1 cells were tested for their ability to modulate the binding of [^{125}I]AII to the cells (Table 1). Pretreatment of the cells for 1 h at 37°C with EGF, IGF-1, insulin or transforming growth factor β_1 (TGF-β_1) had little or no effect on the subsequent binding of [^{125}I]AII to the cells at 4°C. However, pretreatment of the cells with TPA induced a large (2.7-fold) increase in [^{125}I]AII binding.

Table 1
Effect of various agents on the binding of [^{125}I]AII to RIE-1 cells

Addition		Specific [^{125}I]AII binding (% control ± S.E.M.)
No pretreatment		
Control		100 ± 4
EGF	(20 ng/ml)	96 ± 1
IGF-I	(100 ng/ml)	105 ± 4
Insulin	(1 μg/ml)	108 ± 5
TGF-β_1	(1 ng/ml)	100 ± 10
TPA	(30 nM)	274 ± 7
Cycloheximide pretreatment		
Control		99 ± 10
TPA	(30 nM)	244 ± 9

Cells were pre-treated with vehicle or cycloheximide (10 μg/ml) for 1 h at 37°C as indicated. Test agents were then added for a further 1 h at 37°C and [^{125}I]AII binding was determined. Each value represents the mean of triplicate determinations (± S.E.M.).

The time course of the effect of two different concentrations of TPA on [^{125}I]AII binding is shown in Fig. 1a. At the higher concentration tested (300 nM), TPA stimulated a rapid increase in [^{125}I]AII binding which reached a peak value (3.6-fold over basal) 30 min after addition of the agent. In a total of twenty experiments, TPA stimulated a mean maximal 3.0-fold increase in [^{125}I]AII binding. The maximal increase of binding (Fig. 1a) was sustained for a further 1 h, but thereafter decreased to the basal value by 6 h. In contrast to this biphasic response, treatment of the cells with 3 nM TPA increased [^{125}I]AII binding to a plateau value (1.7-fold over basal) at ~2 h which was sustained until the end of the experiment at 6 h.

The treatment period (6 h) required to reverse the increase of [^{125}I]AII binding induced by 300 nM TPA (Fig. 1a) correlated with the TPA treatment period (4–6 h) required to down-regulate immunoreactive PKC in RIE-1 cells (Fig. 1b). This rapid down-regulation of immunoreactive PKC correlates with down-regulation of PKC activity [14]. The biphasic response of [^{125}I]AII binding to the higher dose (300 nM) of TPA (Fig. 1a) may therefore be explained by an initial activation of PKC (which increases [^{125}I]AII binding), followed by a progressive down-regulation of the kinase which abrogated the initial effect. Results obtained using various concentrations of TPA for short (45 min) and long (6 h) incubations were consistent with the summation of the effects of activation and down-regulation (data not shown).

To confirm the role of PKC in the elevation of [^{125}I]AII binding, RIE-1 cells were depleted of the kinase by prolonged (16 h) incubation with a high dose (300 nM) of TPA. Subsequently, the addition of fresh TPA to these

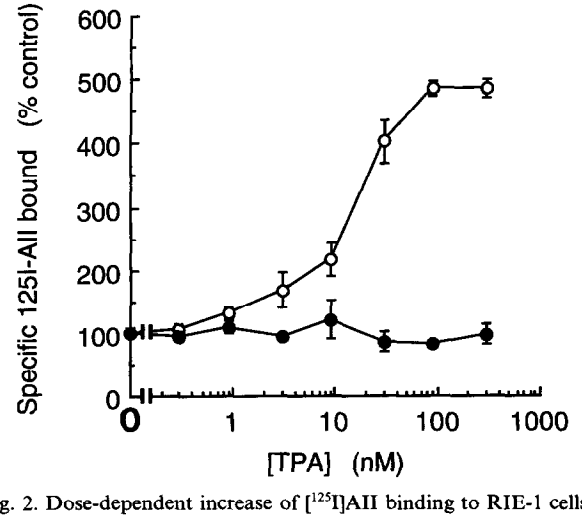

Fig. 2. Dose-dependent increase of [^{125}I]AII binding to RIE-1 cells by TPA. Cells were incubated for 16 h at 37°C with vehicle (○), or 300 nM-TPA (●) to down-regulate PKC. Fresh TPA was then added for 45 min at 37°C and specific [^{125}I]AII binding was determined. Each point represents the mean of triplicate determinations (± S.E.M.).

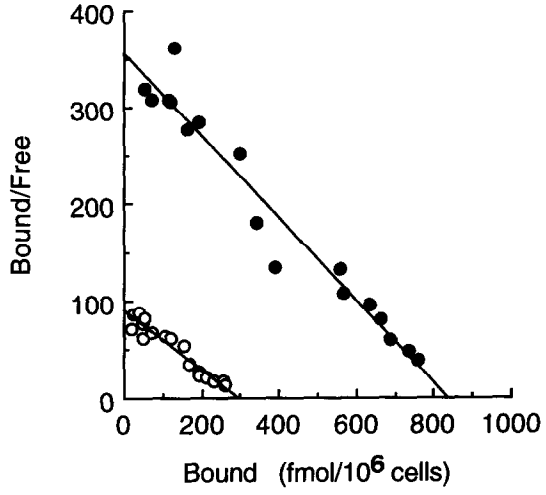

Fig. 3. TPA increases the number of angiotensin receptors on RIE-1 cells. Cells were treated with vehicle (○) or 300 nM TPA (●) for 45 min at 37°C prior to overnight incubation at 4°C with [^{125}I]AII in the concentration range 0.1–20 nM. After removing an aliquot of medium for the determination of free [^{125}I]AII, specific [^{125}I]AII binding was determined. The data is presented as a Scatchard [14] plot. Each point represents a single determination.

PKC-depleted cells failed to induce any increase in binding, whereas the agent increased binding to untreated cells (Fig. 2). This result confirms that the effect of TPA on [^{125}I]AII binding is mediated via PKC, and not via any other action of the agent.

The TPA-induced increase of [^{125}I]AII binding to RIE-1 cells could result from an increase in the affinity and/or number of angiotensin receptors on the cells. The concentration-dependent binding of [^{125}I]AII to control and TPA-treated cells was therefore investigated. Scatchard [15] analysis of the data is shown in Fig. 3. The plot for the TPA-pretreated cells is a linear slope essentially parallel to that obtained using untreated cells, indicating that PKC increased the number of angiotensin receptors on RIE-1 cells (by 2.9-fold in this experiment) without having any significant effect on their binding affinity.

Selective antagonists, which are used to classify angiotensin receptors into AT_1or AT_2 subtypes [16,17], were employed to characterise the TPA-upregulated angiotensin receptors on RIE-1 cells (Table 2). Incubation of untreated RIE-1 cells with the AT_1-selective antagonist, DuP 753, inhibited the majority (84%) of specific [^{125}I]AII binding, indicating that most angiotensin receptors on these cells are the AT_1 subtype. In the experiment shown, DuP 753 also inhibited 46% of specific [^{125}I]AII binding to the TPA-upregulated receptors. The mean inhibition by DuP 753 of specific [^{125}I]AII binding to TPA-upregulated receptors was 69% (range 46–86%; $n = 4$). Hence, a significant proportion of the TPA-upregulated angiotensin receptors are the AT_1 subtype.

Interestingly, the remaining specific [^{125}I]AII binding sites on untreated (16%) and TPA-treated (54%) RIE-1 cells were insensitive to both DuP 753 and the AT_2-selective angiotensin receptor antagonist, PD 123319. These results suggest that, in addition to AT_1 receptors, RIE-1 cells may also express 'atypical' angiotensin receptors (similar to those described recently for Neuro-2A murine neuroblastoma cells [18,19]), and that these 'atypical' receptors are also up-regulated by TPA.

Because of its rapid time course, it seems unlikely that the up-regulation of [^{125}I]AII binding to RIE-1 cells results from increased angiotensin receptor synthesis. This was confirmed by investigating the effect of the protein synthesis inhibitor, cycloheximide, on the TPA-mediated increase of [^{125}I]AII binding (Table 1). At the concentration of cycloheximide used (10 μg/ml), the agent inhibited 95% of the incorporation of [^{35}S]methionine into total RIE-1 cell proteins (data not shown). However, the same concentration of the agent failed to inhibit the TPA-mediated increase of [^{125}I]AII binding to RIE-1 cells (Table 1).

4. Discussion

The existence of negative feedback regulation exerted by PKC on many G protein-coupled receptors to limit their activation is well documented [20]. In addition to an uncoupling of receptors from their effector systems, a common feature of this regulation is a reduction in receptor number and/or affinity [20]. In contrast, a PKC-induced up-regulation in receptor number, as reported here for the AT_1 angiotensin receptors on RIE-1 cells, is less common. Indeed, PKC-mediated up-regulation of angiotensin receptors has only previously been reported in primary cultures of rat neurones [21,22]. In common with RIE-1 cells, angiotensin receptor up-regulation in rat neuronal cultures was rapid and independent of protein synthesis, although a doubling of receptor number was offset by a 50% reduction in their affinity [21,22]. However, rat neuronal cultures are heterogeneous (containing 10–15% non-neuronal cells [23]) and express both AT_1 and AT_2 angiotensin receptors [24]. Consequently,

Table 2
Inhibition of [^{125}I]AII binding to TPA-treated RIE-1 cells by selective antagonists

	Specific [^{125}I]AII bound (% control ± S.E.M.)		
	Control	TPA-pretreated	
		Total	Up-regulated
Control	100 ± 3	181 ± 5	81 ± 6
DuP 753 (10 μM)	16 ± 1	60 ± 3	44 ± 3
PD 123319 (10 μM)	101 ± 5	175 ± 1	74 ± 5

Cells were treated with vehicle or 300 nM TPA for 45 min at 37°C. [^{125}I]AII binding was then determined in the presence of the indicated antagonists. The calculated binding of [^{125}I]AII solely to the TPA-upregulated receptors is also shown. Each value represents the mean of triplicate determinations (± S.E.M.).

the observed action of TPA on [^{125}I]AII binding to these cultures may represent a summation of the effects of PKC on these two different cell types.

In principle, there are several possible mechanisms whereby PKC could rapidly increase the binding of [^{125}I]AII to AT_1 receptors on RIE-1 cells. Up-regulation may result from an increased availability (to extracellular ligand) of a pool of preformed but previously 'cryptic' AT_1 receptors residing at or near the plasma membrane. Such receptors might usually exist in a conformation that renders them refractory to extracellular ligand, but undergo a PKC-mediated conformational change to allow ligand binding. Alternatively, PKC may induce a translocation to the cell surface of preformed AT_1 receptors from an intracellular compartment, analogous to the increased recycling of transferrin receptors to the plasma membrane induced by TPA in Chinese hamster ovary fibroblasts [25] or macrophages [26].

A direct effect of PKC on AT_1 angiotensin receptors is plausible since the intracellular carboxy-terminal tail of this receptor contains several potential serine/threonine phosphorylation sites [27]. Although PKC-mediated phosphorylation at any of these sites has yet to be demonstrated, the kinase is known to phosphorylate the intracellular domains of several other G protein-coupled receptors [28].

In conclusion, PKC increases the binding of [^{125}I]AII to RIE-1 cells, due (in part) to an up-regulation in the number of AT_1 angiotensin receptors. The rapidity of this response, and its independence from protein synthesis, suggest an increased availability (to extracellular ligand) of preformed, but previously 'cryptic', AT_1 angiotensin receptors. RIE-1 cells will provide a useful model system in which to further investigate this possibility.

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