Volume 251, number 1,2, 27–30 FEB 07337 July 1989

Tumor promoter 12-O-tetradecanoylphorbol 13-acetate inhibits *mas*/angiotensin receptor-stimulated inositol phosphate production and intracellular Ca^{2+} elevation in the 401L-C3 neuronal cell line

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Received 1 May 1989

Stimulation of *mas*-oncogene transfected 401L-C3 cells by angiotensins leads to the production of inositol phosphates. This response shows dose dependence, and has an apparent rank order of potency angiotensin III ⩾ angiotensin II ⩾ angiotensin I. Preincubation with 12-O-tetradecanoylphorbol 13-acetate, for 5 min, significantly diminishes both inositol phosphate and intracellular [Ca^{2+}] responses to angiotensins, without affecting those stimulated by the endogenous bradykinin receptor. Incubation of 401L-C3 cells with either phorbol ester or angiotensins leads to elevation of intracellular pH, implying that *mas*/angiotensin receptor stimulation itself leads to protein kinase C activation. These results suggest the operation of a negative feedback loop specific for the *mas*/angiotensin receptor signalling pathway, and which may be essential in defining the final biological output response to this receptor stimulation.

Mas-oncogene/angiotensin receptor; Inositol phosphate; Ca^{2+} intracellular; Tumour promoter

1. INTRODUCTION

The *mas*-oncogene is predicted to encode a protein having 7 potential transmembrane α-helices, and as such, to belong to a family of sensory receptors including the opsins, α- and β-adrenergic, muscarinic acetylcholine and serotinergic receptors [1–3]. Amongst receptors thus far sequenced, the *mas*-oncogene itself shows greatest sequence identity with the substance K receptor [4], leading to the prediction that its product would be a mitogenic peptide receptor capable of stimulating inositol phosphate and $[Ca^{2+}]_i$ responses [3]. Expression of the *mas*-oncogene either transiently, by injection of synthetic *mas*-mRNA into *Xenopus* oocytes, or stably, by transfection into 401L-C3 neuronal cells, generated a novel sensitivity to angiotensins, not present in either uninjected oocytes or the parental NG115-401L cell line [5]. In the 401L-C3 cells stimulation of the *mas*/angiotensin receptor leads to mobilisation of intracellular Ca^{2+}, and also to the initiation of DNA synthesis. The parental NG115-401L cell line responds to stimulation of the endogenous bradykinin receptor by the production of inositol phosphates and a concomitant elevation of intracellular [Ca^{2+}] [6]. However, neither the parental, nor the *mas*/angiotensin receptor-transfected 401L-C3 cells exhibit mitogenic responses to bradykinin. This has led to the suggestion that the *mas*/angiotensin receptor may not actually operate via inositol lipid signalling, but may exert its effects by coupling to a qualitatively different intracellular signalling pathway [7]. Here we report that stimulation of the *mas*/angiotensin receptor, in 401L-C3 cells, leads to the accumulation of inositol phosphates, to elevation of intracellular [Ca^{2+}] and pH, and that these processes may be sensitive to regulation by protein kinase C.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2. MATERIALS AND METHODS

2.1. *Cell culture*

401L-C3 cells were routinely cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal-calf serum (FCS, v/v) and passaged by trypsinisation (0.05%, w/v) in divalent cation free phosphate buffered saline. For inositol lipid experiments cells were plated at a density of 3×10^4 cells/cm^2 on 6 well plates and grown for 3 days before the addition of radiolabel for the final 24 h: 1 μCi of *myo*-[^{3}H]inositol (12.8 Ci/mmol; NEN) in *myo*-inositol free DMEM (Gibco) supplemented with 5 μM *myo*-inositol and 5% dialysed FCS, per well. For population fluorescence measurements 1×10^4 cells were plated on sterile 11 mm × 22 mm glass coverslips in 0.2 ml DMEM (5% FCS, v/v) supplemented 1 h later with 0.8 ml DMEM (5%, FCS, v/v) and used 3 days later.

2.2. *Assay for [^{3}H]inositol phosphate production*

The labelling medium was removed and each well was washed twice with modified Hank's buffered saline (mHBS: 1 mM $CaCl_2$, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM $MgSO_4$, 137 mM NaCl, 44 mM glucose, 20 mM Hepes, pH 7.4) and preincubated in this medium with the addition of 10 mM LiCl, for 15 min at 37°C, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) being added for the final 5 min as appropriate. The preincubation medium was then replaced with fresh prewarmed medium containing the appropriate drugs, and 10 mM LiCl. Incubations were terminated by addition of an equivalent volume of ice-cold 10% (v/v) $HClO_4$; after 5 min on ice, the supernatant was removed (EDTA added to a final concentration of 10 mM) and neutralised by addition of an excess of (1.2:1, v/v) tri-*n*-octylamine/1,1,2-trichloro-trifluoroethane (Freon) (1:1, v/v, see [8]). Solble [^{3}H]inositol metabolites were then separated by ion-exchange chromatography on 1 cm columns of Dowex AG1X8 resin (formate form, Biorad), with batchwise elution as follows; free inositol with 2 × 5 ml of distilled water; glycerophoshoinositol-containing fraction with 2 × 5 ml of 60 mM sodium formate/5 mM disodium tetraborate; mixed inositol mono-, bis-, tris-, and tetrakis-phosphates were eluted by addition of 1.2 M ammonium formate/0.1 M formic acid. The activity in each fraction was determined by liquid scintillation spectrophotometry. Elution positions were verified by co-chromatography with known radiochemical standards.

2.3. *Measurement of intracellular [Ca^{2+}] in cell populations*

Coverslips coated with cells, as described above, were incubated in DMEM containing 2 μM fura-2/acetoxy methyl ester for 45 min at 37°C, washed, and put into mHBS at 37°C and allowed to cool to room temperature for maintenance prior to use.

Before addition of agonists, coverslips were placed across the diagonal of a 0.5 cm path length quartz cuvette, containing 1.5 ml of mHBS with either 1 mM $CaCl_2$. The cuvette was then placed with the coverslip at 60° to incident light, and such that light fell directly onto the cells without passing through the glass coverslip, in a Perkin-Elmer 3000 fluorescence spectrophotometer with a cuvette holder thermostatically maintained at 37°C. Excitation and emission wavelengths of 340 nm and 500 nm, respectively, were chosen so that increasing $[Ca^{2+}]_i$ gives an increase in fluorescence intensity. Calibration and calculation of $[Ca^{2+}]_i$ was as previously described [9].

2.4. *Measurement of intracellular pH in cell populations*

Coverslips prepared as described above were incubated for 30 min at 37°C with 5 μM BCECF/AM [9]. Cells were then washed with mHBS and maintained in this buffer at room temperature until ready for use (the omission of bicarbonate from the original Hanks balanced salts recipe allowing observation of changes in pH_i, without any contribution to intracellular buffering from the HCO_3^{2-}/Cl^{2-} exchange). Coverslips were loaded into a cuvette containing 1.5 ml of mHBS and positioned as described above. Excitation and emission wavelengths of 485 nm and 530 nm, respectively, were chosen so that a rise in intracellular pH gave a corresponding increase in fluorescence intensity.

3. RESULTS AND DISCUSSION

Stimulation of *mas*-oncogene-transfected 401L-C3 cells, prelabelled with [^{3}H]inositol, with angiotensins I, II or III results in the accumulation of inositol phosphates (fig.1a). As previously reported for Ca^{2+} responses in 401L-C3 cells, both AIII and AII show greater potency than equivalent doses of AI (AIII significantly different from AI, $p < 0.05$ as determined by Student's *t*-test (two tailed)). Further, the stimulation of [^{3}H]Ins P accumulation by angiotensin III (AIII) is dose dependent (fig.1b) giving a maximal increase in Ins P_{1-4} of around 150 ± 5% of control (typical basal and stimulated values; 600 ± 40 cpm and 970 ± 100 cpm, respectively). Preincubation of cells with 200 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA), for 5 min, has no significant effect on basal [^{3}H]inositol phosphate levels, but totally abolishes the accumulation stimulated by 100 nM angiotensin III (fig.1c: AIII alone different from AIII after TPA; $p < 0.05$ by Student's *t*-test). This effect is specific to the accumulation stimulated by the *mas*/angiotensin receptor, as preincubation with 200 nM TPA has no significant effect on the [^{3}H]Ins P response stimulated by bradykinin in these cells (fig.1a and c; maximal stimulated accumulation around 280 ± 25% of control, typical basal and stimulated values; 600 ± 25 cpm and 1600 ± 300 cpm, respectively; bradykinin alone not significantly different from bradykinin after TPA; $p > 0.05$ by Student's *t*-test (two tailed)).

401L-C3 cells, prelabelled with the intracellularly trapped, fluorescent Ca^{2+} indicator fura-2, show a basal intracellular $[Ca^{2+}]$ of around 100 ± 60 nM, stimulation by angiotensin II leads to a rise in $[Ca^{2+}]_i$ to a peak value of around 500 ± 140 nM after 15 s, returning to near basal levels some 60 s

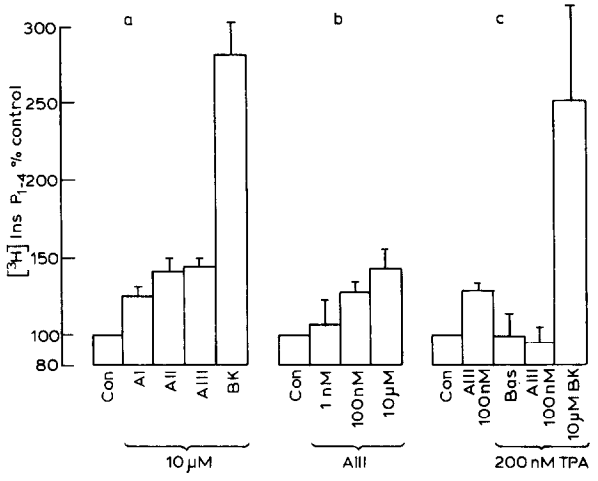

Fig.1. Inositol phosphate production stimulated by angiotensins: (a) relative potency; (b) dose dependence; (c) inhibition by TPA. TPA preincubation, 5 min; all agonist incubations, 30 min. Data are presented as mean ± SE, from 3 to 4 separate experiments, each performed in triplicate.

later. Pretreatment with TPA (200 nM, 5 min) does not alter basal $[Ca^{2+}]_i$ but leads to a substantial diminution of the peak $[Ca^{2+}]_i$ level attained 15 s after stimulation with 100 nM angiotensin II (fig.2).

The magnitude of the angiotensin-stimulated inositol phosphate responses seen in 401L-C3 cells are similar to those described in primary cultures of both arterial smooth muscle [10] and renal mesangial cells [11] endogenously expressing angiotensin receptors. Endogenous angiotensin responses also show sensitivity to inhibition by protein kinase C activators [11,12] and, in renal mesangial cells, it appears that it is the angiotensin receptor itself which is the target of protein kinase C [13]. Further, the presence of potential protein kinase C phosphorylation sites in the *mas*/angiotensin receptors predicted protein product support its role as a point of regulation of receptor effector coupling.

Stimulation of a phospholipase C coupled angiotensin receptor, leading to the concomitant production of inositol phosphates and diacylglycerol, may itself lead to activation of protein kinase C. One convenient marker of protein kinase C stimulation is provided by the rise in intracellular pH following activation of the Na^+/H^+ antiporter [9,14]. Thus in 401L-C3 cells loaded with the, intracellularly trapped, fluorescent pH indicator dye BCECF addition of 200 nM TPA leads to an increase in fluorescence corresponding to an increase in intracellular pH. A similar increase in BCECF fluorescence is recorded in response to 1 μM angiotensin III, implying that stimulation of the *mas*/angiotensin receptor may itself lead to activation of protein kinase C and the Na^+/H^+ antiporter (fig.3).

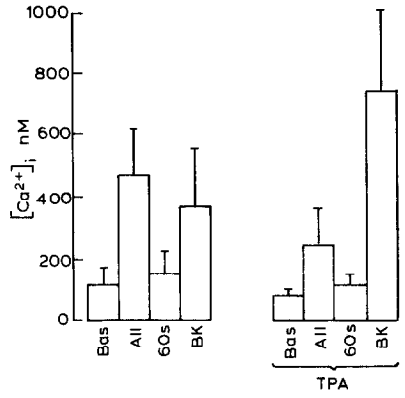

Fig.2. Preincubation with 200 nM TPA diminishes peak $[Ca^{2+}]_i$ response to 1 μM angiotensin II but not that to successive stimulation with 1 μM bradykinin. Data are mean ± SE from at least 5 separate determinations.

The activation of protein kinase C in response to angiotensin stimulation of the *mas*-oncogene may provide a negative feedback loop by which the activity of this receptor is regulated. Such feedback loops have been reported to limit responses to other mitogenic inositol phosphate and Ca^{2+} mobilising receptors, such as, the V1 vasopressin

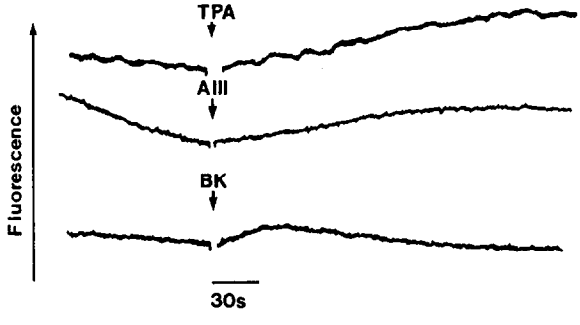

Fig.3. TPA (200 nM), angiotensin III (1 μM) and bradykinin (1 μM) stimulate elevation of intracellular pH as indicated by an increase in BCECF fluorescence. Traces are representative of at least 5 separate determinations.

and bombesin receptors in Swiss 3T3 fibroblasts [15]. The rapid activation of such a feedback process could slow the initial production of inositol phosphates and diacylglycerol after *mas*/angiotensin receptor stimulation, resulting in the slower kinetics observed for both Ca^{2+} [5] and pH responses when compared with those stimulated by bradykinin. This process may also be responsible for the more limited production of inositol phosphates in response to angiotensins than bradykinin in the 401L-C3 cells. A similar disparity in the ability of mitogenic and non-mitogenic receptors to stimulate inositol phosphate production has been reported in human fibroblasts responding to thrombin or bombesin [16], leading to the suggestion that the operation of such tightly regulated negative feedback loops is important in generating mitogenic responses. One possible explanation for this may be found in the recent observations that protein kinase C activation following an initial mitogenic stimulus may actually inhibit cell cycle progression [17,18], thus it may be characteristic of mitogenic inositol phosphate mobilising receptors that their activity is damped once their initial cellular signalling event is completed.

Acknowledgement: T.R.J. was supported by an MRC partnership award with S.K.&F. (UK) Ltd.

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