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Insulin-like growth factor 1 and insulin reduce epidermal growth factor binding to Swiss 3T3 cells by an indirect mechanism that is apparently independent of protein kinase C

Anthony N. Corps and Kenneth D. Brown

Department of Biochemistry, AFRC Institute of Animal Physiology & Genetics Research, Babraham, Cambridge CB2 4AT, England

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Insulin-like growth factor 1 and insulin reduced the binding of ^{125}I-labelled epidermal growth factor (^{125}I-EGF) to Swiss 3T3 cells by 15–20% at 37°C, but not at 4°C. Scatchard analysis indicated that IGF-1 and insulin affected the higher-affinity component of EGF binding, an effect previously associated with the activation of protein kinase C. However, the inhibition of ^{125}I-EGF binding by IGF-1 and insulin was increased, not reduced, when the cells were treated with high concentrations of phorbol esters to down-modulate protein kinase C. We suggest that IGF-1 and insulin activate a protein kinase with similar or overlapping specificity to that of protein kinase C.

Insulin-like growth factor 1; Insulin; Protein kinase C; Epidermal growth factor receptor

1. INTRODUCTION

Swiss mouse 3T3 fibroblasts provide a useful model for studies of cell proliferation, mechanisms of growth factor action, and interactions between the receptors for different growth factors [1]. A diverse group of mitogens for Swiss 3T3 cells stimulate the breakdown of PIP_2 to IP_3 and diacylglycerol [2,3]. Diacylglycerol is a potent activator of protein kinase C [4,5], and the mitogens that induce PIP_2 breakdown stimulate several responses which can also be initiated by the activation of protein kinase C by phorbol esters or exogenous diacylglycerol. These include the phosphorylation of a protein of M_r 80000 ('80K protein') [6], and the phosphorylation of the EGF receptor on Thr^{654} [7,8] which reduces the affinity of the receptor for EGF and hence decreases the cellular binding of ^{125}I-EGF [3,8–12].

The IGFs and insulin are an important second group of mitogens for Swiss 3T3 cells, giving a marked synergistic stimulation of DNA synthesis when used in combination with other mitogens, including agents which stimulate PIP_2 hydrolysis and/or activate protein kinase C [1]. Furthermore, the component of mitogenic stimulation induced by insulin is not sensitive to the down-modulation of protein kinase C by chronic treatment with phorbol esters [13,14]. These results indicate that protein kinase C is not a principal mediator of the mitogenic actions of IGF-1 or insulin, but they do not preclude the possibility that IGF-1 or insulin might activate protein kinase C to a limited extent, as suggested for insulin in BC3H-1 cells [15,16]. We have repeatedly observed that IGF-1 and insulin cause a small reduction in the binding of ^{125}I-EGF to Swiss 3T3 cells. Here, we characterize this effect and demonstrate that it is unlikely to be due to an activation of protein kinase C.

Correspondence address: A.N. Corps, Dept of Biochemistry AFRC Institute of Animal Physiology & Genetics Research, Babraham, Cambridge CB2 4AT, England

Abbreviations: EGF, epidermal growth factor; IGF, insulin-like growth factor; IP_3, inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; PDBu, phorbol 12,13-dibutyrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate

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2. EXPERIMENTAL

2.1. *Materials*

Dulbecco's modified Eagle's medium, antibiotics, trypsin and newborn calf serum were obtained from Flow Laboratories. Genetically engineered [Thr^{59}]IGF-1 (manufactured by Amgen), Na^{125}I, and [20(n)-^{3}H]PDBu were obtained from Amersham International. Mouse EGF and ^{125}I-EGF (spec. act. 900 Ci/mmol) were prepared as in [17,18]. PDBu, TPA and porcine insulin were obtained from Sigma and bombesin was from Bachem (England). Peptide hormones and phorbol esters were stored frozen as concentrated stock solutions in 10 mM HCl and dimethyl sulphoxide respectively, and were diluted in binding medium immediately before use. The equivalent volume dilutions of dimethyl sulphoxide did not affect the binding of ^{125}I-EGF.

2.2. *Methods*

Stock cultures of Swiss 3T3 cells were maintained and passaged as described [18]. For experimental use, cells were seeded into 24-well cluster trays or 35-mm dishes (Nunc) and grown for 7-10 days, by which time they were confluent and quiescent. Cells in which protein kinase C had been down-modulated were obtained by adding 1 μM PDBu (or 200 nM TPA) to the culture medium for the last 48 h prior to the experiment. The cells were then washed three times at 37°C with binding medium [18] before performing ^{125}I-EGF binding assays at 37°C (75 min, for maximal binding) or 4°C (150 min) as in [18,19]. Total and non-specific binding (in the presence of a 200-fold excess of unlabelled EGF) were about 4 and 0.2% respectively, of the total radioactivity.

The binding of [^{3}H]PDBu to Swiss 3T3 cells was performed by a method based on that in [20]. The cells were incubated for 30 min at 37°C in binding medium [18] with [^{3}H]PDBu at the concentrations shown in section 3, in the absence or presence of 1 μM TPA to determine non-specific binding. At the end of the incubation the cells were washed three times with PBS (pH 7.4) at 4°C; the bound radioactivity was released using 0.1 M NaOH to solubilize the cells, mixed with acidified scintillation fluid and determined using a Packard scintillation counter.

3. RESULTS

When Swiss 3T3 cells were incubated at 37°C with IGF-1 (100 ng/ml) or insulin (10 μg/ml), the binding of ^{125}I-EGF was reduced to 82 ± 2 or 85 ± 2% respectively of control values (mean ± SE, $n = 10$). Dose-response curves indicated that the maximal effect on ^{125}I-EGF binding was obtained at concentrations of insulin above 1 μg/ml and of IGF-1 greater than or equal to 100 ng/ml (not shown). In contrast to the effects observed at 37°C, IGF-1 and insulin had no effect on the binding of ^{125}I-EGF to Swiss 3T3 cells incubated at 4°C (fig.1a), indicating that the inhibition at 37°C was indirect rather than competitive.

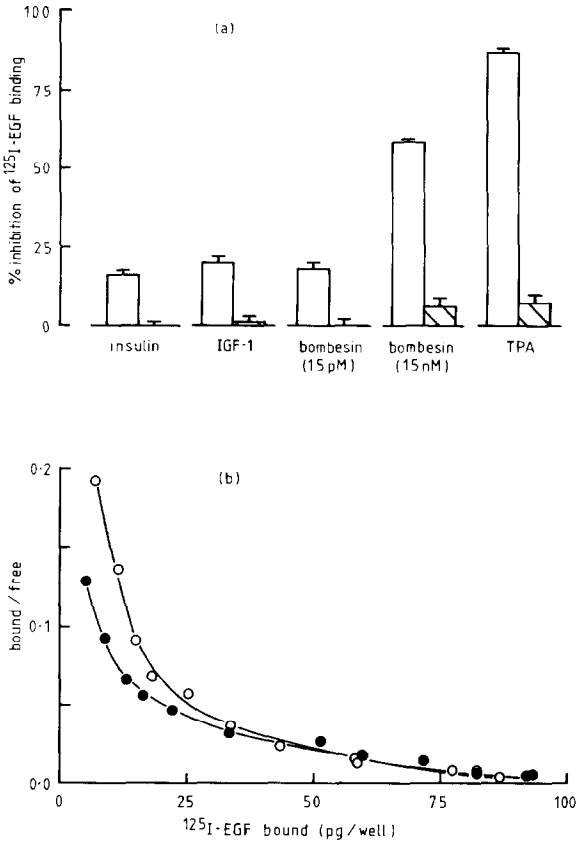

Fig.1. Inhibition of ^{125}I-EGF binding to Swiss 3T3 cells at 37 and 4°C. (a) Cells were incubated at 37°C (open columns) or 4°C (hatched columns) with ^{125}I-EGF (1 ng/ml) and the following additions: insulin, 10 μg/ml; IGF-1, 100 ng/ml; bombesin, 15 pM and 15 nM; TPA, 10 nM. The reduction of specific binding from that in control cells was then determined: mean ± SE, $n = 3$. (b) Scatchard analysis of the binding of ^{125}I-EGF (0.1-50 ng/ml) at 37°C in the absence (○) or presence (●) of insulin (10 μg/ml). At the end of the incubation a sample of supernatant was removed to determine the free ^{125}I-EGF, and the specific binding was then determined. Similar results were obtained in two other experiments using IGF-1 and insulin respectively.

When the inhibitory effects of IGF-1 or insulin on ^{125}I-EGF binding were measured as a function of ^{125}I-EGF concentration, the reduction in binding was greatest at low concentrations of ligand. Scatchard plots of the data (fig.1b and not shown) were consistent with a significant ($p < 0.05$) reduction in the number of higher-affinity receptors ($K_d \sim 4 \times 10^{-11}$ M), with no significant changes in total number of receptors or affinity of either class of receptor.

The addition of bombesin or phorbol esters resulted in maximal inhibition of ^{125}I-EGF binding of about 60 or 80–85%, respectively (figs 1,2), consistent with the large effect of these activators of protein kinase C on the higher-affinity component of ^{125}I-EGF binding, demonstrated previously by Scatchard analysis [3,9,10]. The maximal inhibition induced by IGF-1 or insulin was approximately equal to that induced by up to 15 pM bombesin (fig.1a), 60 pM TPA, or 0.5 nM PDBu (fig.2a). A plot of the specific binding of [^{3}H]PDBu to Swiss

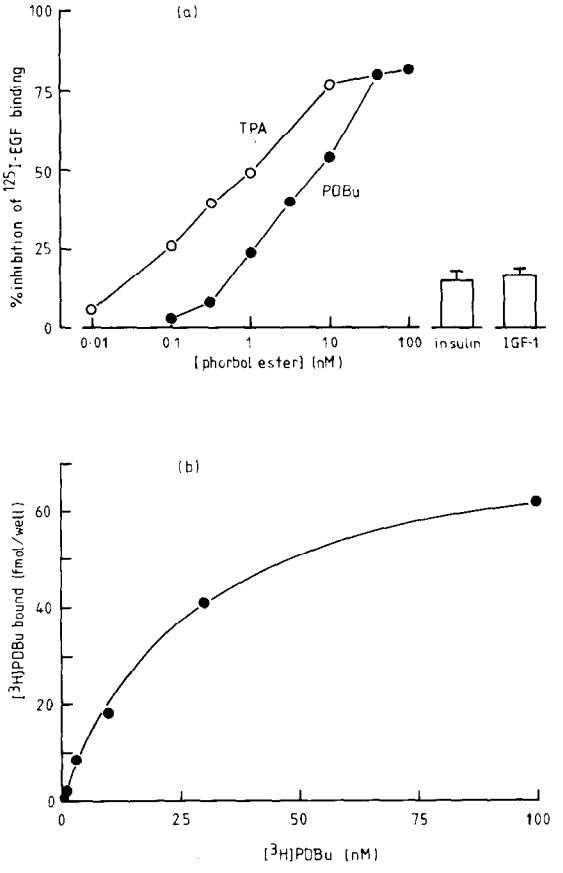

Fig.2. Inhibition of ^{125}I-EGF binding to Swiss 3T3 cells by phorbol esters, and the binding of [^{3}H]phorbol dibutyrate to Swiss 3T3 cells. (a) Cells were incubated for 75 min at 37°C with ^{125}I-EGF (1 ng/ml) and TPA (○) or PDBu (●) at the concentrations indicated. The reduction of specific binding from that in control cells was then determined: means of duplicates. The inhibition by insulin (10 μg/ml) and IGF-1 (100 ng/ml) in the same experiment is also shown: mean ± SE, $n = 3$. (b) Cells were incubated for 30 min at 37°C with [^{3}H]PDBu at the concentrations indicated, and the specific binding was determined.

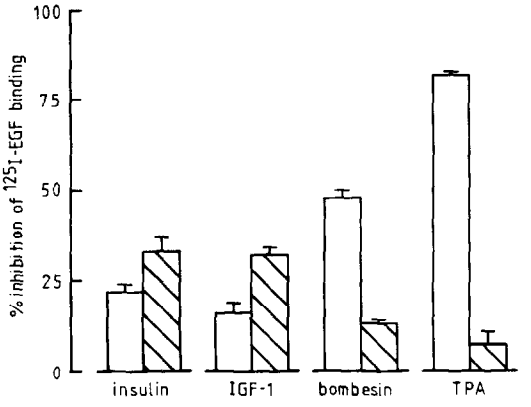

Fig.3. Effect of protein kinase C down-modulation on the inhibition of ^{125}I-EGF binding to Swiss 3T3 cells. Untreated cells (open columns) and cells in which protein kinase C had been down-modulated as described in the text (hatched columns) were incubated for 75 min at 37°C with ^{125}I-EGF (1 ng/ml) and the following additions: insulin, 10 μg/ml; IGF-1, 100 ng/ml; bombesin, 15 pM; TPA, 10 nM. The reduction of specific binding from that in control cells was then determined: mean ± SE, $n = 3$.

3T3 cells, assumed to represent binding to protein kinase C [4], is shown in fig.2b. The dose of PDBu (0.5 nM) which was equivalent to IGF-1 or insulin in its effect on ^{125}I-EGF binding occupied less than 2% of the maximum available protein kinase C. In contrast, maximally effective concentrations of bombesin had effects similar to concentrations of PDBu which occupied 20–30% of protein kinase C.

The mechanism of IGF-1 or insulin action on ^{125}I-EGF binding could be dissociated from the activation of protein kinase C by the down-modulation of protein kinase C using prolonged pretreatment of the cells with high concentrations of phorbol esters. In such cells, the inhibitory effects of bombesin or phorbol esters on ^{125}I-EGF binding were substantially reduced (fig.3). In contrast, the inhibitory effects of insulin or IGF-1 actually increased (fig.3) to a level greater than the residual inhibition by bombesin or TPA.

4. DISCUSSION

Several observations suggest that IGF-1 and insulin do not activate protein kinase C in Swiss 3T3 cells. IGF-1 and insulin synergise in mitogenic stimulation with agents that stimulate protein

kinase C [1,13,14]; insulin does not induce the phosphorylation of the 80K protein substrate for protein kinase C [6]; neither IGF-1 nor insulin increases the breakdown of PIP_2 to IP_3 and diacylglycerol which could activate protein kinase C [21,22], but they potentiate the bombesin-stimulated production of IP_3 [21] whereas activation of protein kinase C is inhibitory [23]. If a subpopulation of protein kinase C that is unusually resistant to down-modulation (as described in BC3H-1 cells [24]) mediates the effects of IGF-1 and insulin on ^{125}I-EGF binding to Swiss 3T3 cells, then it is also unusual in not mediating an action of TPA on ^{125}I-EGF binding. It therefore seems more likely that the effects of IGF-1 and insulin on ^{125}I-EGF binding are mediated by a mechanism independent of protein kinase C.

Recently, it was shown that platelet-derived growth factor is able to inhibit ^{125}I-EGF binding to cells in which protein kinase C has been down-modulated [25]. This response appeared not to be due to activation of any residual protein kinase C, but was mediated by another kinase which is able to phosphorylate the EGF receptor on Thr^{654}. If the inhibition of ^{125}I-EGF binding by IGF-1 and insulin reported here also proves to be associated with the phosphorylation of this residue, then this would suggest that in the cascade of phosphorylation responses activated by different growth factors through their receptor tyrosine kinases [26], there are protein kinases of similar or overlapping specificities to that of protein kinase C. Such a network of protein kinases would provide a mechanism for the convergence of different signalling systems onto the pathway of cellular activation.

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