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INHIBITION OF INSULIN RECEPTOR BINDING BY A23187: SYNERGY WITH PHORBOL ESTERS

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The ionophore A23187 inhibits the binding of insulin on U-937 monocyte-like cells which had been induced to differentiate by 1α, 25-$(OH)_2$ cholecalciferol, while it remains inactive on the undifferentiated cells. A 50% reduction of the specific binding of ^{125}I-insulin is observed within 20 to 30 min at 37°C. The effect is obtained at 10^{-7}M to 10^{-6}M A23187. It is reversible in 60 min at 37°C. A suboptimal concentration of the ionophore potentiates the inhibitory action of phorbol esters on insulin binding.

The function of the insulin receptor is regulated by various hormones or cellular signalling mechanisms. Insulin itself is a major and permanent controlling factor, through the well-known phenomenon of down-regulation of the receptors (1). In addition, phorbol esters (2,3) probably through the stimulation of protein kinase C (4) and β-adrenergic agonists, through a cyclic AMP-dependent process (5-7), induce a rapid inhibition of the binding ability of insulin receptors.

In the present work, we investigated the role of cytosolic calcium in regulating the binding of insulin. Our study has been conducted in the human monocyte-like cell line U-937 (8). These cells possess insulin receptors which are sensitive to the inhibitory effect of phorbol esters (2,9). We took advantage of the recently described property of 1α, 25-$(OH)_2$ cholecalciferol to induce the differentiation of U-937 cells to monocytes, while it increases the binding of insulin (10). Our data demonstrate that cytosolic calcium is able to regulate the function of the hormone receptors in the differentiated U-937 cells, while it remains without effect in the uninduced cells. This phenomenon is synergistic with the action of phorbol esters on the binding of insulin.

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MATERIALS AND METHODS

U-937 cells were cultured in suspension in plastic culture flasks (Nunc, Denmark) in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Eurobio, France), 100 units/ml penicillin and 50 µg/ml streptomycin, at 37°C in a 5% CO_2-95% air humidified atmosphere. Differentiation was induced by incubation with 1α, 25-$(OH)_2$ cholecalciferol (10^{-8}M, during five days) and controlled as already described (10-15). For the binding assays the cells were harvested by centrifugation, washed twice and resuspended in the incubation medium. The binding of insulin was measured at 37°C or at 4°C, as previously reported (2). Degradation of the tracer was assessed by measuring its ability to bind to insulin receptors of human placental membranes (2). At 37°C, it was 15% of the unbound radioactivity at 45 min and was not affected by addition of the ionophore. In all experiments viability of the cells was tested by the trypan blue exclusion method and was higher than 95%.

^{125}I-(A 14)-monoiodinated human insulin was from Amersham (United Kingdom), monocomponent porcine insulin from Novo Industries (Copenhagen, Denmark) phorbol esters and ionophore A23187 from Sigma (St Louis, MO, USA). The 1α, 25-$(OH)_2$ cholecalciferol was a gift from Hoffman-La Roche (Switzerland). Stock solutions of the phorbol esters (1.6 x 10^{-4}M) and A23187 (10^{-3}M) in acetone, as well as 1α, 25-$(OH)_2$ cholecalciferol (10^{-4}M) in ethanol were kept at -20°C and diluted as indicated in the incubation medium.

RESULTS AND DISCUSSION

Treatment of undifferentiated U-937 cells with A23187 had no effect on insulin binding (Fig.1), while it induced a decrease in the cells which had been induced to differentiate by 1α, 25-$(OH)_2$ cholecalciferol. The inhibition of the hormone binding was rapid: it was detectable in 5 min and was complete

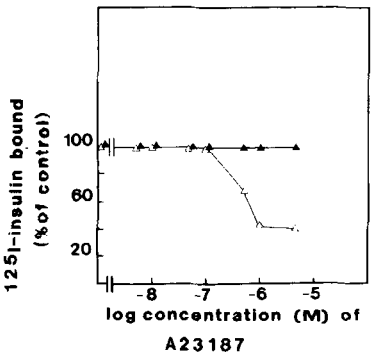
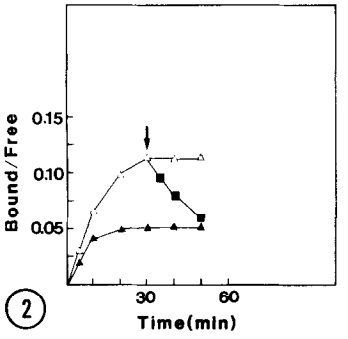


Fig.1. Dose response of the effect of A23187 on ^{125}I-insulin binding - Control (▲) and differentiated (△) U-937 cells were incubated in duplicates for 30 min at 37°C in the presence of the indicated concentration of A23187. ^{125}I-insulin was then added and the specific binding was measured after a further incubation at 37° during 45 min (see Methods). Results are expressed as a percentage of the control (acetone-treated) cells.



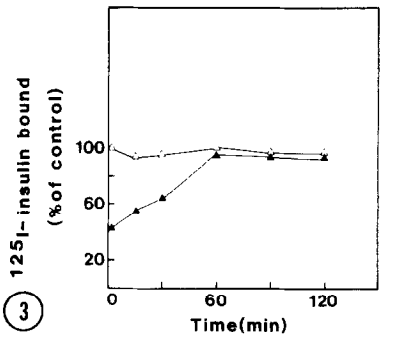


Fig.2. Time course of the effect of A23187 on ^{125}I-insulin binding - Differenciated U-937 cells were incubated in duplicate with 4 X 10^{-11}M ^{125}I-insulin in the presence (▲) or absence (△) of 10^{-6}M A23187 at 37°C. The radioactivity specifically bound to the cells was measured (see Methods) at the indicated time intervals. At 30 min (arrow) 10^{-6}M A23187 was added to some of the controls and the assays were performed at various times thereafter (■).

Fig.3. Reversibility of the effect of A23187 on insulin binding - Differentiated U-937 cells were incubated in RPMI-1640 medium and 10% fetal calf serum in sterile polypropylene tubes for 30 min at 37°C in the presence (▲) or absence (△) of 10^{-6}M A23187. At zero time the cells were washed three times and incubated at 37°C in the above medium in the absence of the calcium ionophore. Duplicate aliquots were removed at the indicated time intervals and the specific binding of ^{125}I-insulin was measured after incubation at 4°C during 180 min (see Methods).

in 20 min (Fig.2). In these experiments the ionophore was present during both the preincubation and the ^{125}I-insulin binding assay. Similar results were observed however, when the cells were preincubated at 37°C with A23187, washed twice in ice-cold medium and the binding of ^{125}I-insulin was subsequently measured during 3 hours at 4°C in the absence of A23187 (data not shown). If both the preincubation and the binding assay were performed at 4°C the ionophore remained ineffective. The action of A23187 on differentiated U-937 was reversible: after preincubation and washing out of the ionophore, the insulin binding ability of the treated cells increased to the control level in 60 min (Fig.3). Our data demonstrate that the insulin receptors are sensitive to the action of A23187 and that this phenomenon shows a high degree of cell specificity. Similar results have been observed by Myers and Siegel (16): stimulation of phospholipase activity by calcium ionophore was obtained in U-937 cells only after they had been induced to differentiate. In the human promyelocytic cell line HL60, differentiation was also re-

quired for the ionophore to express its secretory stimulating effect (17), despite the fact that it was able to transport calcium as efficiently in uninduced cells as it did in induced ones. These findings, therefore, imply that the appearance of factors beyond the step of the increase of intracellular free calcium is required for the expression of the action of the A23187 ionophore.

The cell specificity of the phenomenon reported in our study is also demonstrated by previous work in pancreatic acini (18,19). In this cell type the increase of intracellular calcium inhibited the binding of epidermal growth factor and of insulin-like growth factor II, while it had no effect on insulin receptors.

In several experimental situations involving various cellular functions, synergistic effects of phorbol esters and calcium ionophore have been demonstrated (review in 20 and 21). In the U-937 cell line the phorbol esters inhibit acutely and reversibly the binding of insulin (2,9). This effect was also observed after the induction of differentiation by $1\alpha,25\text{-}(OH)_2$ cholecalciferol (Fig.4). Moreover, in differentiated cells, the calcium ionophore at 10^{-7}M, which was inactive by itself on the binding of the hormone, clearly potentiated the effect of the phorbol ester (Fig.4).

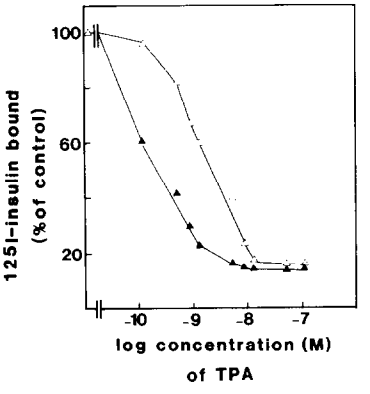


Fig.4. Synergistic effect of A23187 and phorbol esters - Differentiated U-937 cells were incubated in duplicate for 45 min at 37°C with 4 X 10^{-11}M ^{125}I-insulin in the presence of the indicated concentrations of 12-O-tetradecanoyl phorbol 13-acetate (TPA) and in the presence (▲) or absence (△) of 10^{-7}M A23187. Radioactivity specifically bound to the cells was measured (see Methods) and results expressed as a percentage of the control (acetone-treated) cells.

The mechanism of the action of A23187 on insulin receptors remains to be elucidated. The acute inhibition of the binding of the hormone might be explained by a decrease either in receptor affinity or in receptor number. Such an effect has been described with the phorbol esters which are able to induce a rapid internalization of transferrin receptors (22,23). Attempts to evaluate the above concepts by analyzing the kinetics of ^{125}I-insulin binding in control *versus* ionophore-treated cells have proved to be difficult, because of the curvilinearity of Scatchard plots (1) which complicates the interpretations.

The increase in intracellular calcium elicited by A23187 induces several cellular processes by activating the calmodulin-dependent protein-kinases (24). We speculate that these kinases phosphorylate the insulin receptor in differentiated U-937 cells and trigger the inhibition of the hormone binding. If this hypothesis is confirmed, it will add a fourth agent able to induce the phosphorylation of insulin receptor, the other ones being insulin itself (25-28), phorbol esters (29) and cyclic AMP (7). It should be stressed, however, that in some situations, high concentrations of A23187 stimulate nonspecifically protein kinase C (4). In our conditions, we don't have direct evidence to disprove this possible pitfall. Nevertheless, a strong argument against it is the fact that the effect of the phorbol esters (through the kinase C) exists in both the control (2,9) and the differentiated U-937 cells, while the action of A23187 is observed only in the latter.

In conclusion, in this study we demonstrate that the insulin receptors become sensitive to the inhibitory action of the calcium ionophore A23187 during the differentiation of the human monocyte-like U-937 cells induced by 1α,25-$(OH)_2$cholecalciferol. This action is synergistic with the effect of the phorbol esters on the binding of the hormone. Thus, cytosolic calcium is able to modulate the function of membrane receptors for epidermal growth factor (18,19) and insulin-like growth factor II (19) in pancreatic acini, as well as for insulin (this study). We suggest that this effect is mediated by the Ca^{++}-binding protein calmodulin and that the insulin receptors are a target of four different kinases: the receptor-linked kinase (25-28), the protein kinase C

(29), the cAMP-dependent kinases (7) and the calmodulin-dependent kinases.

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