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Down-regulation of phorbol diester binding to NG115-401L neuronal cells is dependent on structure, concentration and time

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The down-regulation of [^{3}H]PDBu binding to a neural cell line, NG115-401L, has been examined in response to two biologically active phorbol diesters, PDBu and PMA. Chronic treatment with PDBu or PMA causes a concentration- and time-dependent loss of specific [^{3}H]PDBu binding. The action of PMA is biphasic with respect to both concentration and time dependence.

Phorbol ester; Protein kinase C; Phorbol diester binding; Down-regulation; (Neuronal cell line)

1. INTRODUCTION

It is now widely recognised that the high-affinity binding site of tumour-promoting and pro-inflammatory phorbol diesters may correspond to the intracellular enzyme PKC [1]. Activation of this kinase produces a variety of biological effects, including actions in neurones [2–4].

In several cell types, long-term exposure to phorbol diesters has been shown to reduce both amount and activity of PKC [5–8]. This down-regulation attracts interest for several reasons: (i) it may be elicited by hormone receptor activation in some circumstances [5]; (ii) its mechanism is unknown; (iii) it can be used to produce 'PKC-deficient' cells in the absence of specific, well-characterised PKC antagonists [3]. Recent reports of tissue-specific expression of a number of different protein kinases C [9] highlight the need to characterise such down-regulation in different differentiated cell populations. Here we report that homologous down-regulation of the high-affinity [^{3}H]PDBu-binding site in the neuronal-like cell line NG115-401L is structure-, concentration- and time-dependent.

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Abbreviations: PDBu, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; HGB, Hank's saline supplemented with 8 mg/ml glucose and 2 mg/ml BSA

2. MATERIALS AND METHODS

Cells were cultured at 37°C in 8% CO_2 and maintained in DMEM supplemented with 5% FCS and antibiotics. For binding measurements, cells were grown on 24-well plates (5×10^5 cells/well) and cultured for 48 h prior to each experiment. All experiments were conducted at room temperature. The DMEM was aspirated and the cells incubated for 10 min prior to addition of the radioactive ligand. When the cells had been pre-exposed to phorbol esters, the medium was aspirated, the cells washed 3 times with 0.5 ml HGB at 37°C and the third wash left in the well for a further 20 min while the plate was kept at 37°C. In control ex-

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periments this washing procedure was shown to remove 99.5% of previously added [^{3}H]PDBu.

All binding assays were performed with 0.5 ml per well of HGB containing 10 nM [^{3}H]PDBu (12.5 Ci/mol, New England Nuclear) plus its vehicle ethanol (max. 2.5%) with 60 min allowed for equilibration. Non-specific binding was defined in the presence of 20 μM unlabelled PDBu (Sigma). The incubations were ended by aspiration and rapid washing (3 × 0.5 ml ice-cold HGB). Wells were extracted and washed with 2 × 0.75 ml of a 1% Triton X-100 solution containing 1 mg/ml BSA. Pooled extracts were counted in 10 ml Hydrofluor (National Diagnostics); efficiency approx. 41%.

For chronic treatment, phorbols were added from a solution in DMEM plus dimethyl sulphoxide vehicle (max. 1.6%). The concentration and duration of treatment are given in the figure legends.

3. RESULTS

Fig.1 shows that 24 h treatment with PDBu or PMA causes a dose-dependent reduction in specific [^{3}H]PDBu binding. The effect of PMA is biphasic; a reduction to about 50% control binding occurring from 10 to 100 nM, and above 200 nM a complete abolition of binding.

The dose-response relationship to PDBu shows a maximal reduction of about 60% in control binding. The effect of PDBu is also less potent than that of PMA at any given concentration tested. The non-esterified β-phorbol (Sigma), which lacks biological activity [10] and was used here as a control, produced no effect.

Fig.2 shows the time course of the down-regulation to PMA, comparing the results for the two concentrations 20 and 200 nM. Fig.3 shows a graphical transformation of the data for 200 nM PMA. It is apparent that the response to the higher concentration consists of two exponential processes; the rapid phase having a $t_{1/2}$ of 1.5 h and the slow phase a $t_{1/2}$ of 13 h. A slow phase with similar kinetics is also present in the response to 20 nM PMA treatment. The lower concentration has a complex effect at short time points, however, such that at 90 min there is a significant increase in specific binding above control ($P < 0.025$). Thus,

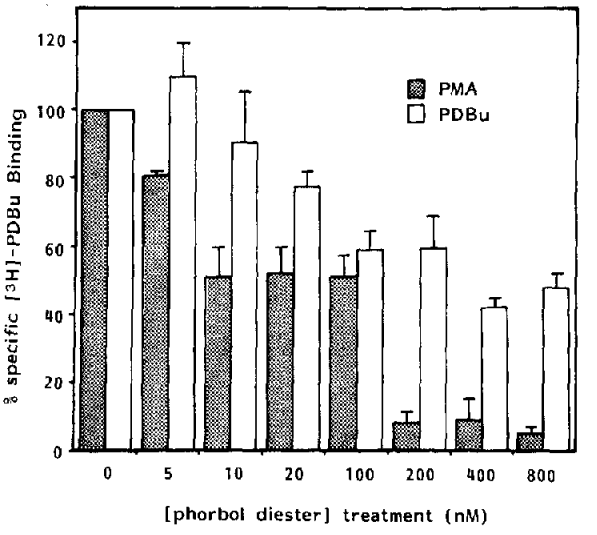

Fig.1. Concentration dependence of down-regulation of specific [^{3}H]PDBu binding by PDBu or PMA treatment. 100% specific binding, defined in the absence of any treatment, represented approx. 87 fmol ligand bound per well. Points shown are the means ± SD of three independent experiments conducted in triplicate samples.

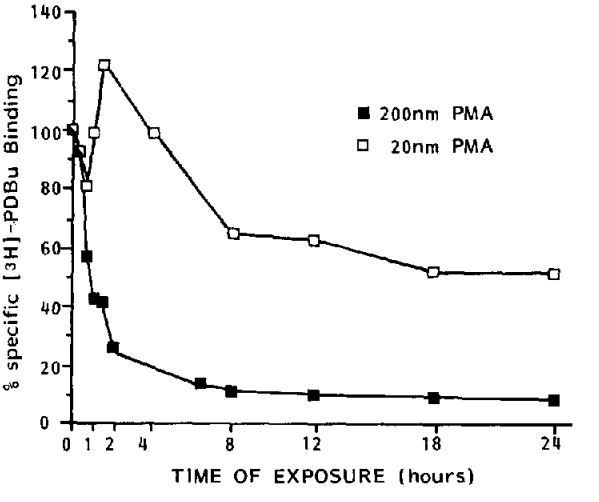

Fig.2. Time course of down-regulation of specific [^{3}H]PDBu binding elicited by PMA treatment. 100% specific binding defined in the absence of any treatment. Points shown are means of three independent experiments conducted in triplicate samples (20, 40, 60, 90 min and 4, 24 h) or two independent experiments of quadruplicate samples (2, 8, 12, 18 h). Standard deviations of the individual points were 1–15%.

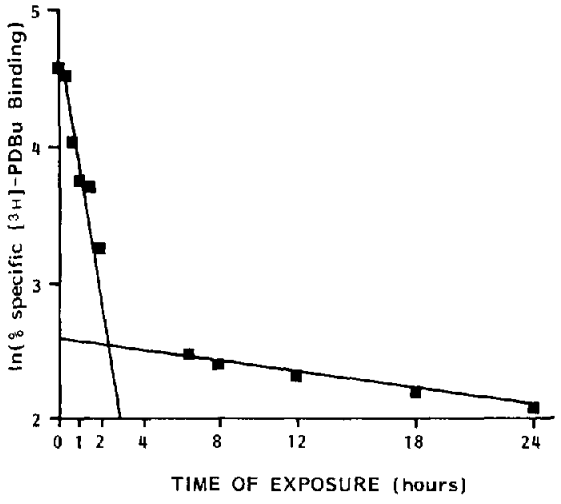

Fig.3. Graphical transformation of data for 200 nM PMA treatments shown in fig.2.

after 4 h at the lower concentration the binding is unchanged from control at 99 ± 1%, whereas after 2 h of 200 nM PMA treatment there has been a striking reduction in the level of binding to only 26 ± 1%.

4. DISCUSSION

These results show a clear distinction between the two most commonly used active phorbol diesters, PDBu and PMA, in eliciting down-regulation. PDBu was developed by Driedberg and Blumberg [11] as a ligand with the optimal ratio of specific binding to non-specific uptake. In contrast, while PMA is known to be the most potent phorbol ester in several biological responses, its high lipophilicity means that it readily partitions into membranes to give a large, non-saturable binding. The potency and extent of the down-regulation in response to these two ligands are quite different. The failure of PBDu treatment to produce complete down-regulation of [^{3}H]PDBu binding has also been reported in Swiss 3T3 fibroblasts [8] and AtT-20/D16V clonal pituitary cells [6]. The lack of effect of β-phorbol confirms that only biologically active phorbols produce down-regulation. Two different concentrations of PMA provoke two different time courses for loss of specific phorbol-binding sites. At the higher concentration (200 nM), the time course can be fitted by two exponential processes (fig.3), whereas at the lower concentration (20 nM), there is an initial rapid loss, followed by a transient increase in binding. In each case the final decay of specific binding appears kinetically equivalent. This suggests that the early loss of binding with sustained application of phorbol diesters may be particularly sensitive to concentration. It is intriguing to consider whether the lower concentration of PMA may more closely mimic the possible regulation of the phorbol binding site in response to the activation of cell surface receptors [5].

The loss of functional PKC following chronic phorbol exposure has been suggested to have two sequential steps; an initial desensitisation with no change in binding properties, followed by a decrease in binding sites [6]. In several cell types, the second stage may be due to an increased rate of PKC degradation [12]. The slower phase of down-modulation, which is present in response to both concentrations of PMA tested, is more likely to be consistent with actual PKC loss. It is an interesting possibility for future investigation that the more rapid events in the alteration of binding could reflect changes in the affinity of the site.

An added complexity to be considered is that recent reports have identified several closely related but distinct forms of protein kinases C [9]. It is likely that any individual clonal cell line may express different protein kinases C with their cognate phorbol diester receptors, thus some heterogeneity in binding characteristics and down-regulation should not be unexpected.

In conclusion we would suggest that to produce fully down-modulated, and presumably maximally PKC-deficient, NG115-401L cells, treatment with PMA (>200 nM, >12 h) should be used in preference to treatment with PDBu.

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