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INHIBITION OF PROTEIN KINASE C MEDIATED SIGNAL TRANSDUCTION BY TAMOXIFEN

IMPORTANCE FOR ANTITUMOUR ACTIVITY

KIERAN HORGAN, ERYL COOKE, MAURICE B. HALLETT and ROBERT E. MANSEL
Department of Surgery, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

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Abstract—Recent studies have demonstrated tamoxifen inhibition of the enzyme protein kinase C (PKC) *in vitro*. The aim of this study was to investigate the effects of tamoxifen on PKC function in intact human cells. As PKC activates the neutrophil oxidase mechanism the neutrophil was chosen as an experimental model to assess PKC–tamoxifen interaction in these experiments. Neutrophils from healthy volunteers were separated by centrifugation through Ficoll Hypaque. Two separate parameters of oxidase activation; oxygen consumption and reactive oxygen metabolite production were monitored by a Clark electrode chamber and luminol dependent chemiluminescence respectively. Neutrophil chemiluminescence was markedly stimulated by 4 Phorbol-12 myristate-13 acetate (PMA). This stimulation was inhibited by tamoxifen; IC_{50} = 6.1 ± 1.6 μM ($\bar{x}$ ± S.E.M.) N = 6. Neutrophil oxygen consumption was similarly stimulated by PMA and inhibited by tamoxifen. The tamoxifen inhibition was not due to cell toxicity as assessment of cell integrity by the exclusion of trypan blue and measurement of intracellular concentrations of ATP showed no significant differences before and after treatment. Tamoxifen also inhibited neutrophil chemiluminescence which was stimulated by oleoyl acetyl glycerol and mezerein excluding interaction with PMA as an explanation of its inhibitory effect. These results are consistent with tamoxifen inhibition of PKC function in intact human cells. This may be central to its antitumour action.

Tamoxifen is a unique anticancer agent. It inhibits the growth of approximately 30% of human breast cancers [1] with minimal side effects [2]. Knowledge of its mode of action would further an understanding of the biology of breast cancer and also enable the development of other effective non-toxic anti-neoplastic compounds. Although tamoxifen has been used for 20 years its mode of action remains unclear. Its ability to bind to oestrogen receptors on breast cancer cells is well documented [3] but the cellular effects of tamoxifen cannot be explained solely by oestrogenic blockade [4]. Clinical studies have also shown that the growth of approximately 25% of receptor negative breast cancers was inhibited [1]. Recent studies have shown that tamoxifen binds to sites distinct from oestrogen receptors [5] but their significance has yet to be established. An extensive review of the literature led Furr and Jordan to conclude that "there is still no satisfactory molecular mechanism" to explain its action [6].

However, recent advances in our understanding of cancer cell biochemistry suggest new possibilities. It is now recognised that the enzyme protein kinase C (PKC) plays a crucial role in tumour promotion [7]. Two recent reports have demonstrated tamoxifen inhibition of PKC activity *in vitro* [8, 9]. It is, therefore, now necessary to determine whether tamoxifen inhibits PKC-mediated processes in intact human cells. It has been established that the neutrophil is a convenient model for the study of PKC activity in tumour promotion [10]. We have also chosen the neutrophil as a source of human cells to assess PKC mediated signal transduction.

MATERIALS AND METHODS

Peripheral blood was obtained from healthy volunteers and the neutrophils were isolated, as previously described [11], by centrifugation through Neutrophil Isolation Medium (Packard United Technologies). 4β Phorbol-12 myristate-13 acetate (PMA), oleoyl acetyl glycerol (OAG), mezerein (Mz) and tamoxifen citrate were purchased from Sigma Chemical Company (Dorset, U.K.). A sample of OAG was also kindly donated by Professor Y. Nishizuka (Kobe, Japan). All agents were dissolved in dimethyl sulphoxide (DMSO). The concentration of DMSO in the cell suspension never exceeded 0.3% (v/v).

Measurement of oxidase activity. Oxidase activation was determined by disappearance of the substrate oxygen and appearance of the products, reactive oxygen species. Oxygen consumption was measured using a Clark electrode chamber (Rank Bros, Bottisham, Cambridge). The production of reactive oxygen metabolites from neutrophil suspensions (approximately 10^6 per ml) containing luminol (11 μM) was monitored by measurement of chemiluminescence using a purpose built thermostatically controlled luminometer, as previously described [12].

ATP measurement. ATP was measured in the supernatant after neutrophil precipitation by perchloric acid (1.8 M) according to the method of Wettermark and Stymne [13]. After neutralisation (potassium carbonate, 3 M, potassium phosphate buffer, 1 M) 10 μl aliquots of the supernatant were

added to luciferin–luciferase in sodium arsenate (0.1 M). Chemiluminescence was monitored and related to a standard curve. In this assay chemiluminescence in the absence of ATP was less than 50 cps and in the presence of 10 pmol was approximately 20×10^3 cps.

RESULTS

Neutrophil chemiluminescence was stimulated by PMA from a resting rate of approximately 100 counts/sec to a maximal rate of approximately 15,000 counts/sec (see Fig. 1). Pretreating the neutrophils with tamoxifen inhibited this response. The possibility that the inhibition was caused by tamoxifen interference with the chemiluminescent assay was eliminated by using a second method for determination of oxidase activation, namely oxygen consumption. The increase in oxygen consumption stimulated by PMA was also inhibited by tamoxifen (see Fig. 2). The inhibition shown with both methods was due to the tamoxifen component since citrate ($\leq 20\ \mu M$) had no effect. Neutrophil integrity as assessed by the exclusion of trypan blue was not reduced by tamoxifen (>99% viability after tamoxifen). There was also no significant difference in intracellular ATP concentrations before and after tamoxifen. (ATP concentration after tamoxifen 90.3 ± 5.7% of untreated level, N = 8. Student's unpaired *t*-test, $P > 0.2$).

Although tamoxifen consistently inhibited PMA stimulated neutrophil chemiluminescence the concentration required showed individual variation. The concentration necessary for 50% inhibition, calculated as illustrated in Fig. 1(b), was 6.1 ± 1.6 μM ($\bar{x}$ ± S.E.M.), range 0.8 μM–12 μM, N = 6.

In order to determine whether the observed inhibition was selective for PMA two other PKC activators, structurally dissimilar to PMA, were used. These agents both stimulated neutrophil chemiluminescence with a time course and magnitude similar to PMA and were also inhibited by tamoxifen (see Fig. 3).

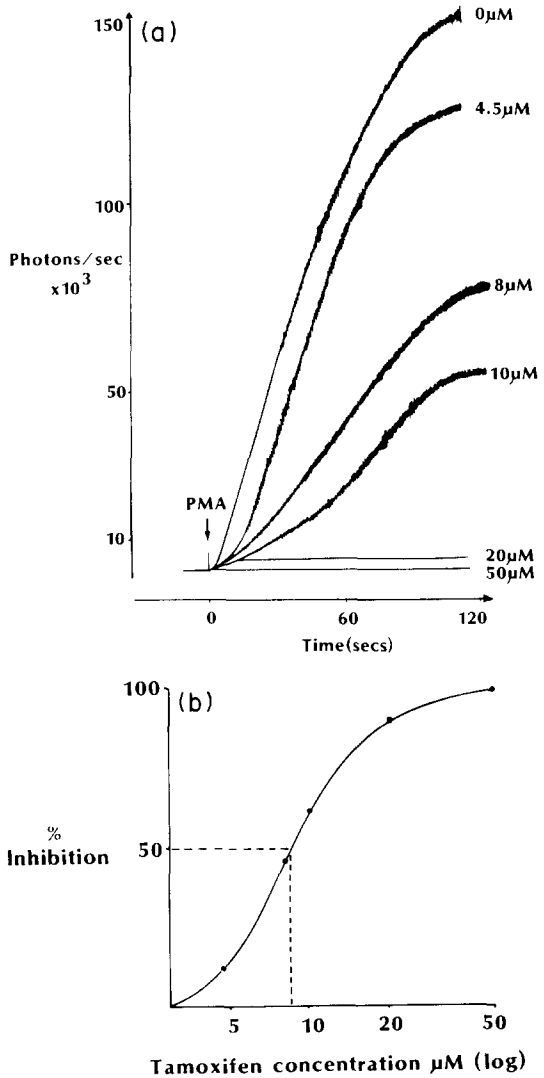

Fig. 1. Inhibition of neutrophil chemiluminescence by tamoxifen. (a) The tracings demonstrate the changes in neutrophil chemiluminescence subsequent to PMA addition (↓) to neutrophils pretreated for five minutes with tamoxifen at the concentrations shown in the right hand column. (b) The percentage inhibition was calculated from the tracing shown in (a) and plotted against tamoxifen concentration. The dotted lines demonstrate the method of calculation of the IC_{50} value.

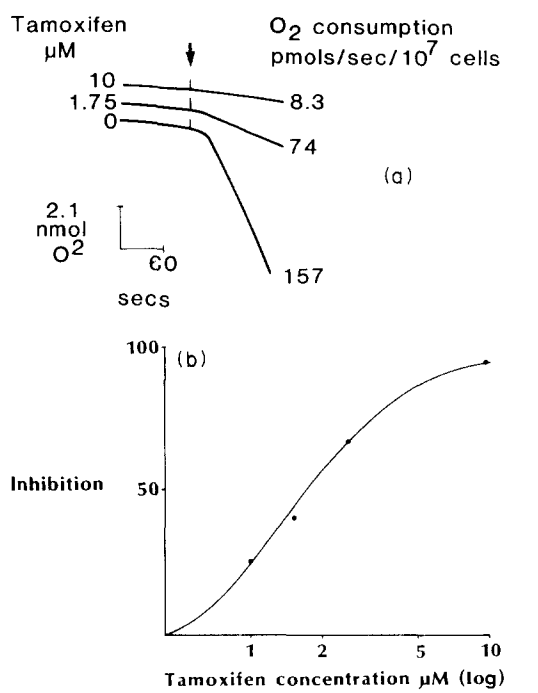

Fig. 2. Tamoxifen inhibition of stimulated neutrophil oxygen consumption. (a) The tracings show the change in oxygen concentration subsequent to PMA addition (↓) to neutrophils pretreated with tamoxifen for five minutes at concentrations shown on the left. The maximum rate of oxygen consumption was calculated from the gradient of the trace. (b) The inhibition of rate of oxygen consumption was calculated as a percentage of the total in the absence of inhibitor and plotted against tamoxifen concentration. The results illustrated are the findings of a typical experiment.

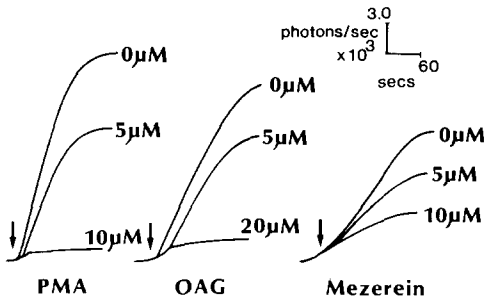

Fig. 3. Tamoxifen inhibition of other PKC stimulators. The tracings of a typical experiment demonstrating neutrophil chemiluminescence in response to PMA (0.1 μg/ml), OAG (2.0 μg/ml) and Mz (0.1 μg/ml). Also shown is the inhibitory effect of 5 min preincubation with tamoxifen at the concentration shown to the right of each tracing.

As neutrophil dysfunction has been rarely reported as a side effect of tamoxifen the neutrophil oxidase response of patients receiving long term tamoxifen was investigated. There was no significant difference in PMA stimulated neutrophil chemiluminescence between the healthy volunteers and the tamoxifen patients ($9.1 \pm 1.2 \times 10^4$ compared to $8.3 \pm 2.9 \times 10^4$ counts/sec/10^6 cells respectively, $\bar{x} \pm$ S.E.M., $N = 5$, $P > 0.8$ Student's t-test).

DISCUSSION

The results demonstrate that tamoxifen inhibits PMA stimulated neutrophil oxidase activation as assessed by the two parameters, oxygen consumption and oxygen radical production. Measurement of intracellular ATP concentration and cell integrity have suggested that this inhibition is not due to toxicity. The inhibitory effect was confirmed in six volunteers with a mean IC_{50} of 6.1 μM. This is higher than therapeutic serum levels [14] but is similar to the concentrations required to inhibit breast cancer growth *in vitro* [15]. There was considerable individual variation in the level of inhibitory response at any given concentration of tamoxifen, the factors responsible for this remaining unknown.

Tamoxifen also inhibited other PKC stimulators, OAG and Mz, suggesting that the inhibition was not caused by tamoxifen–PMA interaction but rather at a cellular level. Interestingly, PMA and Mz are also potent experimental tumour promoters. These findings are therefore consistent with tamoxifen inhibition of PKC activity and signal transduction *in vivo* [16].

There is considerable clinical and experimental evidence that tamoxifen acts by inhibiting breast cancer growth rather than by influencing tumour initiation. Bradbeer *et al.* [17] demonstrated cancer regrowth on cessation of tamoxifen but renewed cancer inhibition on its recommencement in a series of patients with breast cancer who were treated solely with tamoxifen. Osborne *et al.* [18] have shown that tamoxifen inhibited human breast cancer proliferation in athymic mice but that it did not cause tumour regression. The effects of tamoxifen on MCF7 human breast cancer cell cycle kinetics have been documented by Sutherland *et al.* [15], who concluded it possessed "antitumour activity *in vitro* that involves biochemical mechanisms independent of the oestrogen receptor system". These observations could be explained by tamoxifen inhibition of breast cancer cell PKC.

Therapeutic serum levels of tamoxifen are difficult to measure and show considerable variability (0.5 μM–1.3 μM) [14]. Higher concentrations were required to demonstrate inhibition in this paper and elsewhere [15]. It is possible that the local concentration of tamoxifen is critical for its anticancer effect. Binding of tamoxifen to oestrogen receptors may facilitate preferentially elevated local concentrations in the cytoplasm of breast cancer cells. Such a mechanism would explain specific breast cancer PKC inhibition in the absence of other side effects such as neutrophil oxidase inhibition.

The findings from neutrophils have prompted these studies to be extended to breast cells and to the isolated enzyme. A demonstration that tamoxifen inhibits breast cancer PKC would provoke the search for a new range of anticancer agents.

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