CALCIUM AND OESTROGEN INTERACTIONS UPON THE RAT THYMIC LYMPHOCYTE PLASMA MEMBRANE

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SUMMARY. When isolated thymic lymphocytes are equilibrated with a carbocyanine dye the degree of fluorescence is indicative of the potential difference across the plasma membrane. Increments in extracellular calcium concentrations trigger a decrease in fluorescence intensity which represents a membrane hyperpolarisation. This hyperpolarisation cannot be reversed by subsequent chelation of the calcium. Oestradiol and natural oestrogens reduced this calcium-induced potential alteration. These same steroids also blocked the calcium-induced mitotic stimulation apparent in this same cell type.

INTRODUCTION

Elevation of plasma calcium concentrations stimulates thymic lymphopoiesis in the male but not the female rat (1). However, when thymic lymphocytes from both male and female animals were cultured *in vitro* raised extracellular calcium concentrations stimulated proliferation in both groups of cells (1). This lack of response of female lymphocytes to calcium challenge *in vivo* has been attributed to simultaneous presence of oestradiol 17β (1). This steroid also blocked calcium-induced mitogenesis *in vitro* (2-3). The initiation of deoxyribonucleic acid (DNA) synthesis and ultimately division which calcium triggers may involve the movement of this and possibly other ions across the plasma membrane. Thus it has been suggested that oestradiol may act at this superficial level to impede ion fluxes (3). To investigate this possibility a novel technique has been employed in which the transmembrane potential difference is related to the degree of fluorescence in an equilibrated thymocyte and carbocyanine dye system. The interaction of calcium and oestrogens upon ion flux and potential difference across the lymphocyte plasma membrane was studied and found to be related to the effects of these steroids on calcium-induced mitogenesis.

METHODS

To evaluate the action of calcium and oestrogens upon transmembrane potential and mitotic activity a standard thymocyte culture was used in both situations (2,3). Where cells were required for use in the fluorescence system the thymic lymphocytes were suspended in a modified Earle's salts solution containing 7.7mM sodium bicarbonate and buffered to pH 7.3 with 20mM HEPES (Flow Laboratories). However, cells to be used for mitotic assays were maintained in Medium 199 (Burroughs Wellcome) containing no serum or antibiotics and buffered to pH 7.2 with sodium bicarbonate. These latter cultures also contained the metaphase arresting agent colchicine (Ciba Ltd.) at a final concentration of 0.06mM. The percentage of nucleated cells arrested in the so-called colchicine metaphase configuration after six hours incubation was then scored (4) and used as an index of proliferative activity. In both types of experiments cells were incubated in one millilitre aliquots in plastic tubes (Sterilin Ltd.) sealed with non-toxic stoppers. These cultures were then rotated at 40 r.p.m. about their long axes in a roller drum assembly at 37°C until required.

When used for fluorescence studies thymocytes at a final cell density of 4×10^7 cells.ml^{-1} were dispensed into a prewarmed quartz cuvette and placed in an Aminco-Bowman spectrofluorimeter. The apparatus had been modified to facilitate the heating of the cuvette to 37 degrees centigrade and also to permit the gentle stirring of the cell suspension. To the cuvette containing three millilitres of cells was added 60 microlitres of the dye, 3, 3'-dipentyloxacarbocyanine iodide, to give a final concentration of 2μM, and the system allowed to equilibrate. The dye used was the generous gift of Dr.A.Waggoner (Amherst College, Mass.). The dye was excited at 480 nM and fluorescence monitored at 510 nM via a photomultiplier and plotted on a Beckman x-t recorder. Thus addition of further agents which cause alterations in transmembrane p.d. could be characterised by an alteration in trace height against time on the recording device.

Absolute cell numbers (as assessed using a Coulter ZB1 cell counter) did not alter during either of these incubation techniques. Likewise cell viabilities of 98% (determined using trypan blue dye exclusion) were maintained throughout duration of the experiments.

Valinomycin (Sigma Chemical Co.) and A23187 (Lilly Research Centre) were dissolved in ethanol, as were the steroids used in this study with the exception of the glucuronide derivative of oestradiol, which was dissolved in medium. These reagents were kept as concentrates, such that addition of 10 microlitres to a 1 millilitre culture gave the desired final concentration. These low levels of alcohol have been shown to have no action on mitotic activity or cell survival. EGTA (BDH Ltd.) was dissolved in medium.

RESULTS AND DISCUSSION

When valinomycin was added to cells equilibrated with carbocyanine dye it elicited a marked decrease in fluorescence (Figure 1a) which was indicative of a membrane hyperpolarisation generated by the facilitated movement of potassium down its concentration gradient (5,6). After a 30 minute preincubation in the absence of calcium subsequent addition of calcium to a final concentration of 1.8 mM likewise produced a marked decrease in

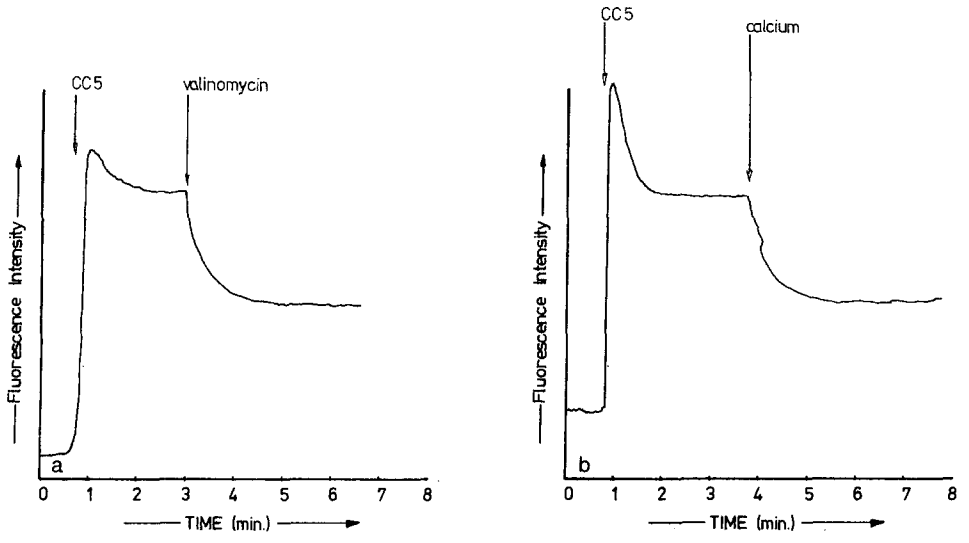

Figure 1a

The effect of adding valinomycin (10^{-5}M) to a stirred suspension of thymocytes, equilibrated with the day CC5, on the fluorescence emission from the system.

Figure 1b

The effect of adding calcium chloride (1.8 mM) to a stirred suspension of thymocytes, equilibrated with the day CC5, on the fluorescence emission from the system.

fluorescence which reached a maximum value within 120 seconds(Fig.1b). Thus calcium addition established membrane hyperpolarisation which is presumably the result of the change in some ion flux. Smaller incremental additions of calcium revealed a progressive increase in hyperpolarisation with the maximum rate of change of fluorescence occurring between 0.2 and 1.1 mM calcium. Maximum values were obtained at 1.8 to 2.0 mM calcium (Figure 2).

This hyperpolarising action of calcium could not have been an artefact of initial calcium deprivation during the preincubation period for when cells were preincubated with various concentrations of calcium further calcium additions still induced hyperpolarisation(Data not shown). The maximum

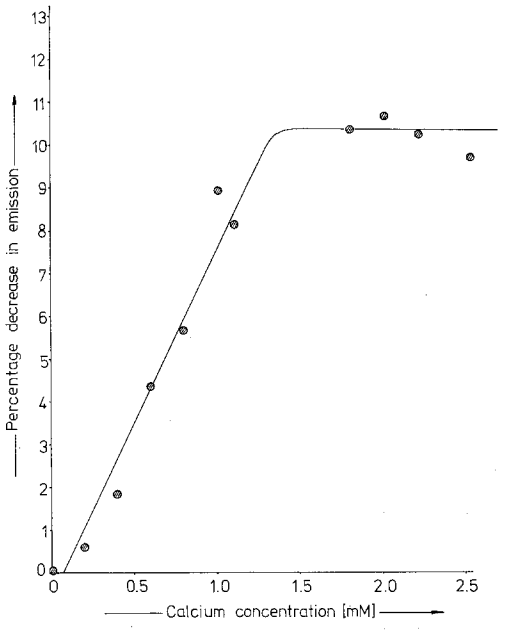

Figure 2

The effect of adding various doses of calcium chloride to stirred thymocyte suspensions, equilibrated with the dye CC5, on the fluorescence emission from the system. Each point is the mean of three individual determinations, and the line fitted by eye.

decrease in fluorescence was again obtained when final calcium concentrations were 1.8 mM.

Of the various divalent cations tested only calcium induced a membrane hyperpolarisation. Magnesium, strontium, zinc, manganese and tin had no effect on fluorescence. Indeed magnesium slightly reduced a calcium-induced fluorescence change when present at high (3 mM) concentrations. Since normal ionised calcium values in plasma may vary between 1.0 and 1.2 mM (Hunt, N.H. personal communication) under different physiological conditions, the highly specific calcium-induced hyperpolarisation of the thymocyte plasma membrane occurring over this concentration range (Figure 2) may well represent a true physiological role for the ion in membrane function.

Once established, a calcium triggered potential change could not be

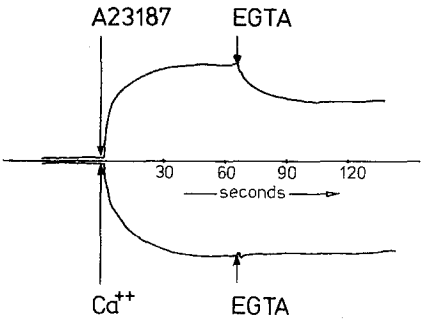

Figure 3

The influence of EGTA (saturated aqueous solution) on calcium-(1.8 mM) and ionophore-induced (10^{-5}M) fluorescence changes.

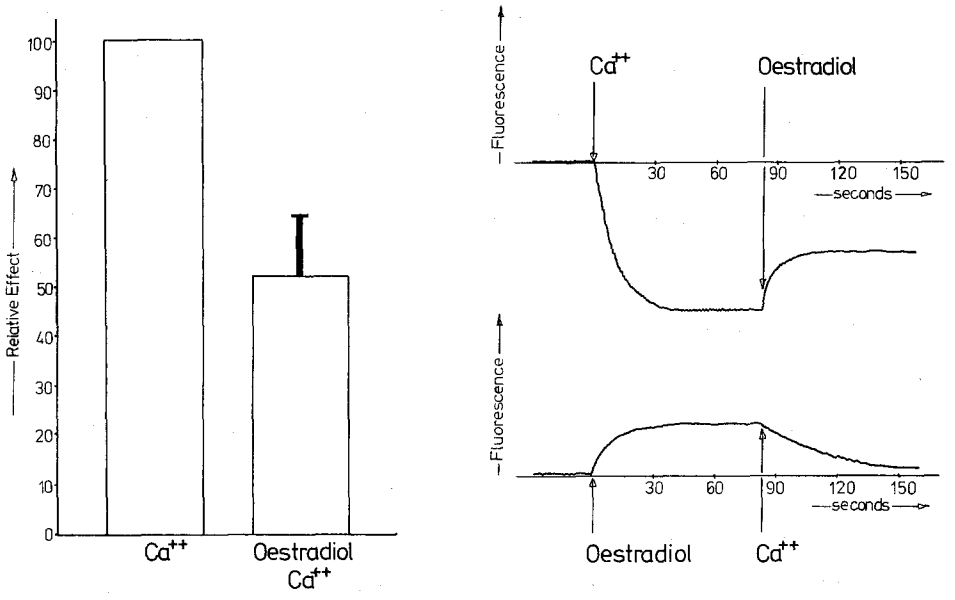

Figure 4

The effect of oestradiol 17β (10^{-5}) on basal and calcium-induced (1.8 mM) fluorescence intensity in thymocyte culture. The column represents the mean of 5 separate determinations and the vertical bar 1 standard error of the mean.

reversed by addition of the calcium chelating agent EGTA (Figure 3). Thus calcium seems to initiate some event which subsequently generates and maintains the observed potential alteration. Certainly it is not related to a simple diffusion of more calcium ions into the cell, for when such a move-

ment is provoked with some specificity by addition of the calcium ionophore A23187 (8) this results in an increase in fluorescence (Figure 3) or hypopolarisation, precisely the opposite effect caused by simple calcium addition. Furthermore the hypopolarisation which followed ionophore addition was at least partially reversed by EGTA addition (Figure 3).

Since oestradiol can inhibit calcium-induced mitogenesis in isolated thymic lymphocyte cultures (2-3) this steroid was added to the cell suspensions in the fluorescence system both before and after the addition of calcium ions. In each case when oestradiol-17β (10 μM) was added to cultures a small but significant hypopolarisation was observed (Figure 4). Furthermore when calcium (1.8 mM) was added to the cultures after the oestradiol, the hyperpolarisation induced by the ion was reduced to some 50% of that in paired cultures containing no steroid (Figure 4). Thus oestradiol blocks both calcium-induced mitogenesis and hyperpolarisation. It is not established however whether the hyperpolarisation and the associated ion fluxes which calcium causes are related to or essential for this ion's ability to recruit cells into their DNA synthetic phase. In an attempt to provide a partial answer to this question a range of steroids was examined to see whether an ability to inhibit calcium-induced mitogenesis was always associated with a capacity to block calcium-induced hyperpolarisation.

Several other oestrogenic molecules also had the ability to inhibit calcium-induced proliferation (Table 1). A superficial analysis of these structures reveals that only 18 carbon steroids possessed this property whilst 19 carbon androgens and 21 carbon progestogens were without any activity. It is also a feature of the anti-mitotic steroids that they possess an aromatic A ring which might represent an important feature of structure activity. However two synthetic derivatives of oestradiol, the 3-methyl ether of oestradiol and the 3-methyl ether of 17-ethyl oestradiol, lacked antiproliferative activity. It is likely therefore that other aspects of structure are important in delineating this action of oestrogens.

Table 1

A comparison of the effect of different steroids upon calcium-induced mitogenesis, calcium-induced hyperpolarisation and resting membrane potential in thymocyte cultures. Steroids were all added to the fluorescence system at 10^{-5}M, and were screened prior to cell addition for any native fluorescence. When studying mitotic activity cells were exposed to steroids at 10^{-9}M. A (+) symbol indicates a positive effect observed on the parameter under test, whilst no change is indicated by /. Where there is a vacant space this agent was not tested.

	SUPPRESS MITOSIS	SUPPRESS Ca^{++} TRIGGER	INCREASE FLUORESCENCE	DECREASE FLUORESCENCE
β-Oestradiol	+	+	+	/
α-Oestradiol	+	+	+	/
β-Oestradiol-3-benzoate	+	+	+	/
Oestriol	+	+	+	/
Oestrone	+	+	+	/
Oestrone-3-sulphate	+	+	+	/
17α-Ethyl-β-oestradiol 3-methyl ether	/	/	+	/
β-Oestradiol 3-methyl ether	/	/	+	/
β-Oestradiol-3-glucuronide	/	/	/	/
β-Oestradiol-17-glucuronide	/	/	/	/
Testosterone	/	/	/	+
Androstene-dione		/	/	/
Androstene-diol	/	/	/	/
Dehydroisoandrosterone	/	/	/	/
Progesterone	/	/	/	/
Cholesterol	/	/	/	/
Anisole		/	/	/

One such parameter may be the groups present on carbon atoms 3 and 17. Since the alpha epimer of oestradiol is equally as effective as the biologically active beta form of the steroid this suggests that the spatial configuration of the carbon 17 hydroxyl is of no importance. Furthermore it would indicate that the interaction being observed is not being mediated through classical cytosol receptors which show little affinity for alpha oestradiol (8,9). It is also possible to replace the carbon 17-hydroxy function of oestradiol with a ketone group with little loss of activity. Also the additional hydroxylation of carbon 16 has no effect upon the ability of the steroid to inhibit calcium-induced mitogenesis. In terms of substitution at the carbon 3 position a sulphate group does not seem to impair function whereas a glucuronide does. This latter observation may relate to the poor lipid solubility of glucuronide forms of steroids (10), an important feature in their excretion. The 3 benzoate derivative of oestradiol blocked calcium-induced mitogenesis whereas the 3 methyl ether derivative did not. Since both compounds are particularly lipid soluble it is unlikely that this property is a primary factor governing mitotic blockade. It is possible that the important features include the general property of lipid solubility, an aromatic A ring and groups at positions 3 and 17 which are capable of some interaction with elements of the membrane structure.

When carrying out a similar structural analysis for the ability to mute calcium-induced fluorescence changes it was apparent again that only carbon 18 oestrogens were active. Thus a close resemblance was noted between the requirements for activity in blocking calcium-induced mitogenesis and hyperpolarisation. Remarkably the biologically inactive alpha epimer of oestradiol was again equally potent, which also suggests a superficial site of action for the steroid. Furthermore oestrone and oestriol possessed some activity as did the 3-sulphate derivative of oestrone. Indeed the only discrepancy between the two groups was the 3 methyl ether derivatives of

oestradiol and the 3-methyl ether of 17-ethyl oestradiol which did block calcium triggered hyperpolarisation but not proliferation.

Parenthetically it might be noted that testosterone induced a small hyperpolarisation, an activity which might indicate some importance for this steroid in membrane function (Table 1) and which was in complete contrast to the small hypopolarisation observed with oestradiol.

It is thus envisaged that calcium interacts with the plasma membrane to provoke an ionic redistribution. Preliminary experiments indicate that potassium ion movements may be particularly relevant in this context. A precedent for this may be the recent report of calcium-induced hyperpolarisation in amphibian erythrocytes, an action mediated by potassium (11). Certainly calcium can stabilise biological membranes by its ability to reduce carbon bond rotations in the lipid palisade components (12). Furthermore under such conditions of decreased membrane fluidity ion transporting ATPases are known to function more efficiently (13). Indeed, it is becoming clear that it is calcium or a consequence of calcium entry into the cell which governs sodium and potassium transport (14,15). It may well be that it is these ion movements which elicit either directly or via intermediate processes, previously unscheduled DNA synthesis. We propose that oestrogens may interfere with this chain of events by interacting with the outer palisade of the membrane (16) in such a way as to prevent calcium exerting this membrane stablising effect.

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