REQUIREMENTS FOR DIVALENT CATIONS BY HORMONAL MITOGENS AND THEIR INTERACTIONS WITH SEX STEROIDS

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SUMMARY. Thymic lymphocyte proliferation in vitro may be modulated by a number of hormones. These hormones may be divided into two distinct functional groups based on their requirement for either calcium or magnesium ions in the extracellular milieu. The action of mitogenic hormones which require calcium ions is blocked by oestradiol, whereas the magnesium-dependent hormonal mitogens are ineffectual in the presence of testosterone.

INTRODUCTION

Mitotic activity in thymic lymphocyte populations both in vivo and in vitro is increased when the concentration of either calcium or magnesium in the extracellular environment is raised (1 - 4). Many hormones can also stimulate mitosis in this cell type (4 - 7). In order to exert their mitogenic action some of these hormones have an obligatory requirement for extracellular calcium ions (5 - 7) whereas others are calcium-independent (8,9). In the presence of oestradiol neither calcium nor the calcium-dependent hormones can express their mitogenic potential (2, 4, 10). However, the stimulatory action of magnesium and the calcium-independent mitogenic hormones is quite unimpeded by this steroid (4). In contrast testosterone blocks magnesium but not calcium-induced mitogenesis (11). The present study was designed to investigate the possible interaction of testosterone with the hormonal mitogens. It revealed that not only can the action of the calcium-independent hormones be blocked by testosterone but also that these agents have an obligatory requirement for magnesium ions.

METHODS

Thymic lymphocyte suspensions were prepared in Medium 199 (Burroughs Wellcome Ltd.) free of serum and antibiotics and buffered to pH 7.2 with sodium bicarbonate. The medium was supplied free of calcium and magnesium ions which were subsequently added to desired levels. One millilitre aliquots containing approximately 5 x 10^7 cells were rotated in sterile, stoppered plastic tubes at 37 degrees centigrade for 6 hours. Proliferative capacity was assessed by addition of colchicine to the cultures at a final concentration of 0.06 mM. Under these conditions lymphoblasts flow linearly into mitosis where they accumulate with time in a quasi-metaphase configuration. They may be readily observed and counted in this state after fixation and staining with Delafields Haematoxylin. These techniques have been described in detail elsewhere (4, 12). Steroid hormones were dissolved initially in 3 millilitres of absolute ethanol, then diluted to 10 millilitres with isotonic saline. This was then further diluted 1:10 with saline so that when 10 microlitres was added to a one millilitre culture the desired level of steroid was obtained. Alcohol alone at this concentration has no effect on mitotic activity. Bovine parathyroid hormone which was kindly donated by the Medical Research Council (Batch number 72/286) was dissolved in a solution comprising 1% sodium acetate, 0.5% L-cysteine hydrochloride and 0.1% crystalline bovine serum albumin which had previously been heated to 56 degrees centigrade to inactivate peptidases. This vehicle has no effect on mitotic activity. All other hormones except glucagon and insulin were dissolved in isotonic saline, whereas the latter were dissolved in unbuffered media. A 10 microlitre aliquot of these concentrates per culture gave the requisite hormone concentration.

RESULTS AND DISCUSSION

The addition of glucagon (10^{-4}M), dopamine (10^{-7}M) or isoprenaline (10^{-6}M) to the thymic lymphocyte suspensions caused a significant stimulation of mitosis whether calcium ions were present or absent (Table 1). In contrast parathyroid hormone (5 MRC units per ml), insulin (100 μU/ml), histamine (10^{-12}M) and acetylcholine$^{\neq}$(10^{-12}M) only stimulated cell division if the culture medium contained calcium (Table 1).

Testosterone (0.1 μg/ml) blocked the action of all the calcium-independent mitogens but had no effect on any of the calcium-dependent mitogens (Table 1). Oestradiol (0.1 μg/ml) only inhibited calcium-dependent mitogens (Table 1). Preliminary experiments had revealed that neither steroid affected the basal level of mitotic activity.

Since oestradiol blocked the mitogenic effect of raised extracellular calcium concentrations and of the calcium-dependent hormones whereas

$\neq$ Cultures containing acetylcholine also contained 10^{-9}M eserine to prevent degradation by acetylcholinesterase.

Ionic or Steroid Status of Medium	MITOGEN							
	NONE	ACETYL CHOLINE 10^{-12}M	PTH 5 MRC units per ml	INSULIN 100 µU/ml	HISTAMINE 10^{-12}M	ISOPRENALINE 10^{-6}M	GLUCAGON 10^{-4}M	DOPAMINE 10^{-7}M
0.6 mM Ca^{++} 1.0 mM Mg^{++}	3.4±0.12	5.6±0.24 *	6.0±0.25 *	6.3 ±0.30 *	6.1±0.18 *	6.1±0.17 *	7.2±0.64 *	5.4±0.14 *
0.0 mM Ca^{++} 1.0 mM Mg^{++}	3.6±0.30	3.2±0.20	3.4±0.44	2.8 ±0.26	3.8 ± 0.60	6.3±0.21 *	6.1±0.46 *	5.9±0.42 *
0.6 mM Ca^{++} 1.0mM Mg^{++} 0.1 µg/ml Oestradiol	3.7±0.30	3.1±0.14	3.7±0.19	3.5±0.18	3.2±0.34	7.3±0.60 *	6.6±0.54 *	5.7±0.39 *
0.6 mM Ca^{++} 0.0 mM Mg^{++}	3.7±0.20	5.8 ±0.25 *	5.5±0.36 *	5.9±0.41 *	5.9 ±0.34 *	3.8 ±0.22	3.2±0.17	3.9±0.50
0.6 mM Ca^{++} 1.0 mM Mg^{++} 0.1 µg/ml testosterone	3.5 ±0.20	5.3±0.20 *	6.0 ±0.33 *	6.1 ±0.29 *	6.9 ±0.38 *	3.9±0.41	3.6±0.40	3.8±0.23

Table 1. The action of steroids and extracellular divalent cations on hormonal mitogens. Results represent the mean percentage of cells in "colchicine-metaphase" at six hours ± one standard error of the mean. Where values are significantly ($p<0.001$) higher than the respective control, this is indicated with an asterisk. Each mean value was derived from between 4 and 22 separate experiments.

testosterone blocked the proliferative response to magnesium (11) and to glucagon, dopamine and isoprenaline (Table 1) it was important to establish whether this latter group of calcium-independent hormones had any requirements for extracellular magnesium ions. Omission of magnesium from the culture medium did in fact prevent the mitogenic response to glucagon, dopamine and isoprenaline (calcium-independent) but signally failed to affect the action of the calcium-dependent parathyroid hormone, insulin, histamine and acetylcholine (Table 1).

This clear dichotomy between calcium-dependent and magnesium-dependent events which ultimately leads to mitosis was further emphasised by the observation that the divalent cations themselves can act separately as mitogens with no dependence or requirement for the other ion. Thus in the absence of calcium raising the magnesium concentration in the medium from the usual 1.0 mM to 2.5 mM significantly increased mitosis from 3.8 $\pm$0.2 to 5.9 $\pm$ 0.3 per cent ($p<0.001$); in magnesium free medium a calcium increment from 0.6 to 1.8 mM was likewise a powerful proliferative stimulus elevating mitosis from 3.8 $\pm$0.2 to 5.6 $\pm$0.4 per cent ($p<0.001$). Omission of calcium, magnesium or both ions from the medium had no effect on basal mitotic activity (Table 1).

The results demonstrate that mitogenic hormones can be separated into two distinct functional groups. Members of the first group (including parathyroid hormone, acetylcholine, insulin and histamine and a number of other agents (5-7)) require calcium ions and are inhibited by oestradiol (Table 1). The second group which contains glucagon, dopamine and isoprenaline (in the present study), and probably adrenaline and certain prostaglandins (8-9), although not having any requirement for calcium ions do need magnesium ions and can be inhibited by testosterone (Table 1).

The two clear-cut routes to mitosis favoured by these hormonal groups parallel other physiological contrasts between certain of the hormones. The opposing actions of adrenaline and acetylcholine and of glucagon and insulin

are well known and have been ascribed to the ability of the antagonists to increase either intracellular cyclic 3',5' adenosine monophosphate (c-AMP) or cyclic 3',5' guanosine monophosphate (c-GMP) concentrations (13). Certainly both adrenaline (8) and glucagon (N.H.Hunt, personal communication) can stimulate adenylate cyclase in plasma membrane preparations from thymic lymphocytes and both these agents and the other magnesium-dependent hormones described above (Table 1) activate this enzyme in other tissues thus provoking an increase in intracellular c-AMP levels (14). Of the calcium-dependent mitogens used only parathyroid hormone has been tested and clearly fails to activate this enzyme in the thymic lymphocyte (15). However, the calcium-dependent hormones acetylcholine, insulin and histamine elevate c-GMP levels in several tissues (16 - 18) whereas this is not a property of the magnesium-dependent hormones used here (19). Since both exogenous c-AMP and c-GMP can both stimulate mitosis in this cell type (7,20) it is reasonable to suppose that these cyclic nucleotides may be major participants along the two mitogenic axes. Thus one might envisage the interaction of parathyroid hormone, acetylcholine, insulin and histamine with plasma membrane receptors which are somehow linked to guanylate cyclase. The obligatory requirement of these mitogens for calcium may be a reflection of the necessity for this ion as a cofactor to active a membranous cyclase enzyme (21). Alternatively calcium may act as an intermediary between the hormone and a soluble guanylate cyclase in the cytoplasm (22). The net effect would be an increase in intracellular c-GMP. A precedent for such a scenario exists in the calcium-dependent mitogen concanavalin A which elevates c-GMP in the thymic lymphocyte (23). Similarly glucagon, dopamine and isoprenaline may only be capable of activating membrane bound adenylate cyclase to increase intracellular c-AMP concentration if extracellular magnesium ions are present as co-activators (24). Alternatively or in addition the divalent cations may be obligatory participants in the mitogenic pathway at points beyond the initial hormone-receptor-cyclase step. Indeed before the

cyclic nucleotides can bring about the common intracellular climax of DNA synthesis and mitosis preliminary results in this laboratory indicate there may be further divalent cation-dependent steps involved. Consistent with an intracellular locus for the action of the divalent cations after stimulation by hormonal mitogens is the influx of calcium and magnesium into some cells provoked by parathyroid hormone (25) and adrenaline (26) respectively.

Finally, although we currently have no idea at what point the steroids interact in the sequence of events which links hormones, ions and cyclic nucleotides to mitosis, the findings present us with new tools with which to dissect the biochemical events controlling cell proliferation.

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