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The promotion of mitosis in cultured thymic lymphocytes by acetylcholine and catecholamines

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Thymic lymphoblasts possess β-adrenergic, dopaminergic and nicotinic receptors. When activated by high concentrations of adrenaline, isoprenaline, dopamine and acetylcholine, magnesium-dependent events are initiated, which culminate in mitosis. These events can be blocked by testosterone. The cells also possess muscarinic and α-adrenergic receptors which respond to low concentrations of acetylcholine, carbamylcholine and noradrenaline. In these cases calcium-dependent, oestradiol-blockable mechanisms are triggered which eventually lead to cell division.

Many hormones and humoral agents influence DNA synthesis and mitotic activity in a variety of mammalian tissues such as skin (Ebling 1974), intestine (Wright et al 1972), liver (Becker 1973), bone marrow (Gordon 1973), thymus (Whitfield et al 1969) and uterus (Martin et al 1973). The reports of neural stimulation of mitosis in the crypts of Lieberkuhn (Tutton 1973) and of increased DNA synthesis in bone marrow stem cells after exposure to cholinergic agents (Byron 1973) suggest that neural regulation of cell proliferation is also feasible. Such a possibility would require neurotransmitter substances to trigger events at the surface of the mitotically competent cell which would elicit DNA synthesis and eventually mitosis. 5-Hydroxytryptamine (Tutton 1974) and adrenaline (Bullough & Laurence 1971; Epifanova & Tchoumak 1963; Tutton & Helme 1974) can certainly influence cell division in epidermal and intestinal tissue and we have briefly alluded to the ability of acetylcholine (ACh) to stimulate mitosis in thymic lymphocytes (Morgan et al 1975; Perris & Morgan 1976). The mitogenic potential of various neurotransmitters and the nature of their interaction with the rat thymic lymphocyte is the subject of this study.

MATERIALS AND METHODS

To investigate the action of ACh and other drugs on the rate of entry of rat thymic lymphocytes into mitosis, thymuses were removed from male albino rats of the Wistar strain (180–200 g) maintained under light ether anaesthesia. The glands were minced in Medium 199 (Burroughs Wellcome Ltd.), which had been previously buffered to pH 7·2 with sodium bicarbonate, and subsequently filtered through moistened cheesecloth to remove any residual aggregates. The resulting cell suspension was then diluted in more media such that 1 ml aliquots pipetted into sterile plastic tubes (16 × 90 mm, Sterilin Ltd) contained approximately 10^7 cells, as assessed using a Coulter ZB 1 electronic cell counter (Coulter Electronics).

The Medium 199 used was supplied free of both calcium and magnesium so that the levels of these ions in the culture could readily be adjusted to the desired concentration by appropriate additions of stock solutions. Normally the calcium and magnesium concentrations were 0·6 and 1·0 mM respectively. The tubes were sealed with sterile caps, placed in a roller drum and rotated at 30 rev min^{-1} about their long axes at 37 °C.

Since only a relatively small part of the thymocyte population (10–20%) is mitotically active (Miller & Osoba 1967), assessment of proliferative activity by measurement of an increase in cell numbers is precluded. The metaphase-arresting agent colchicine was therefore added to cultures to give a final concentration of 0·062 mM. Under these conditions lymphoblasts flow into mitosis and are arrested in a quasi-metaphase configuration where they accumulate linearly with time. After a 6 h incubation in the presence of colchicine a sample was removed from the culture, fixed in neutral phosphate-buffered formalin, and stained with Delafield's haematoxylin. The fraction of cell population arrested in the colchicine metaphase configuration was based on an examination of at least 1000 cells by each of two independent observers.

When the actions of neurotransmitters and a variety of pharmacological antagonists on thymocyte

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proliferation were investigated, these agents were dissolved in 0·9% NaCl (saline) so that when 10 µl was added to the cell suspensions at the start of a 6 h incubation in the presence of colchicine it gave the required final concentration. When the steroids oestradiol-17β and testosterone were employed, they were first dissolved in a minimum quantity of absolute ethanol and then made up to the required concentration with saline before addition to the cultures. Preliminary experiments indicated that this small amount of ethanol had itself no effect on basal mitotic activity or on cell morphology. Unless otherwise indicated all the agonists and antagonists were obtained from Sigma Chemical Co.

RESULTS

When thymic lymphocytes were incubated with a range of ACh concentrations a biphasic stimulation of mitosis was seen at approximately 10^{-9} M and at 10^{-5} M (Fig. 1). On different occasions the position of the peak in mitotic activity seen at the lower concentration was variable. Carbamylcholine, an acetylcholinesterase-resistant analogue of ACh, showed optimal and consistent stimulatory activity at a much lower concentration (10^{-11} M) than did ACh itself (Fig. 1) suggesting that differing rates of neurotransmitter degradation might account for the variability. This notion was reinforced by experiments in which cells were incubated with both ACh and the cholinesterase inhibitor, eserine. The mitotic peak seen at high (10^{-5} M) ACh concentrations was unaffected whilst that seen with the lower concentrations was moved down to a level (10^{-11} M) comparable with carbamylcholine (Fig. 1). At the concentration employed (10^{-9} M), eserine alone had no effect on basal mitotic activity (results not shown).

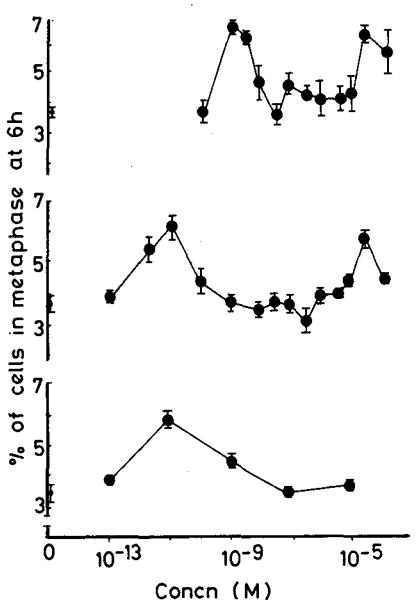

FIG. 1. The influence of ACh (top), ACh + eserine (middle) and carbamylcholine (bottom) upon thymocyte proliferation. Each point represents the mean percentage of cells in metaphase at 6 h ± 1 s.e.m. derived from 4–17 separate experiments.

To investigate this biphasic mitogenic effect of ACh further, susceptibility to the muscarinic receptor antagonist atropine and nicotinic receptor antagonist hexamethonium was tested. Atropine at both 10^{-5} and 10^{-11} M blocked the mitogenic effect of the low concentrations of ACh but had no effect on the mitotic stimulation induced by the high concentration of the neurotransmitter (Table 1). A competitive blockade of a muscarinic receptor is most probable since increasing the ACh concentration overcame the inhibition. Thus in the presence of 10^{-5} M atropine the mitogenic peak normally associated with the low concentration (10^{-11} M) of ACh was moved to the right (10^{-8} M; results not shown). It was also apparent that, when present alone, atropine (10^{-11} M) could itself exert a mitogenic effect. On the other hand hexamethonium (10^{-5} M) only abolished the stimulatory effect of the high concentrations of ACh (Table 1): lower hexamethonium concentrations (10^{-1} M) were without effect on either of the two mitogenic doses of ACh. The activation of either muscarinic or nicotinic receptors on the thymic lymphocyte seems to initiate events which culminate in mitosis presumably via different mechanisms.

Since previous studies (Morgan et al 1975) had indicated that hormonal mitogens operated either via a calcium-dependent, oestradiol-blockable axis,

Table 1. The effect of receptor antagonists, ions and steroids on ACh-induced mitogeneis. Cells were exposed to ACh and the other agents at the concentrations indicated. All cultures also contained the anticholinesterase agent eserine (10^{-9} M) which itself had no effect on mitotic activity. Results are the mean percentage of cells in metaphase at 6 h ± s.e.m. derived from 4–22 separate experiments. Significance higher than the basal control ($P < 0.01$) is indicated as *.

Treatment	Percentage of cells in metaphase at 6 h		
	Control	ACh (5×10^{-5} M)	ACh (10^{-11} M)
No additions	3·5 ± 0·1	6·2 ± 0·4*	6·5 ± 0·5*
Atropine (10^{-5} M)	4·3 ± 0·6	6·1 ± 0·3*	3·5 ± 0·3
Atropine (10^{-11} M)	5·0 ± 0·4*	—	4·0 ± 0·1
Hexamethonium (10^{-5} M)	4·5 ± 0·3	3·0 ± 0·3	6·1 ± 0·4*
Hexamethonium (10^{-11} M)	4·3 ± 0·3	6·8 ± 0 5*	6·9 ± 0·3*
Calcium omitted	3·6 ± 0·3	6·8 ± 0·7*	2·9 ± 0·2
Magnesium omitted	3·7 ± 0·2	3·0 ± 0·2	5·7 ± 0·3*
Oestradiol (0·1 µg ml^{-1})	3·7 ± 0·3	5·4 ± 0·1*	2·9 ± 0·2
Testosterone (0·1 µg ml^{-1})	3·5 ± 0·2	3·1 ± 0·3	6·5 ± 0·4*

or via a magnesium-dependent, testosterone blockable route, it seemed appropriate to investigate the ionic dependence and steroid sensitivity of ACh induced mitogenesis. The action of the high mitogenic concentration of ACh could be abolished by either testosterone addition or magnesium omission (Table 1). The low mitogenic concentration of ACh required calcium to be effective and it could be inhibited by oestradiol (Table 1).

When the physiological antagonist of ACh, adrenaline and its more stable specific analogue isoprenaline were tested on the thymic lymphocyte cultures they too proved to be mitogenic with optimum activity apparent at 5×10^{-6} and 10^{-6} M respectively (Table 2). The effects seemed to be mediated via a β-receptor since the responses were abolished by propranolol but not by the α-receptor blocker phentolamine mesylate. Dopamine likewise stimulated mitosis; a response which could only be specifically impaired by the dopamine receptor blocker pimozide (Table 2). All three agonists exerted their effects via a magnesium-dependent reaction which was itself abolished by testosterone (Table 2). In contrast, very low concentrations of noradrenaline (10^{-12} M) stimulated mitosis in a calcium-dependent, oestradiol-blockable manner. This effect seemed to be exerted via an α-receptor since phentolamine inhibited the response whereas propranolol had no effect (Table 2).

DISCUSSION

In addition to the ability of thymic lymphocytes to divide in response to various systemic and local hormones such as glucagon, adrenaline, parathyroid hormone, antidiuretic hormone, insulin, histamine and prostaglandins (Morgan et al 1975; MacManus et al 1971; MacManus & Whitfield 1970; Whitfield et al 1972) it is now clear (Fig. 1, Tables 1 and 2) they can likewise respond to a variety of neurotransmitters. At first sight the sensitivity to such an array of compounds would suggest a lack of selectivity, mitosis perhaps being the ultimate consequence of non-specific plasma membrane perturbations. However, the present work clearly indicates that the thymic lymphocyte bears upon its surface discrete nicotinic, muscarinic, α- and β-adrenergic and dopaminergic receptors. When these are occupied by the appropriate agonists, events are initiated which culminate in mitosis. These events can be divided into two broad categories. One is magnesium-dependent (and testosterone blockable), the other calcium-dependent (and oestradiol-blockable). Thus activation of nicotinic, β-adrenergic and dopaminergic receptors is linked in some way to magnesium ions whilst activation of muscarinic and α-adrenergic receptors is linked to calcium ions (Tables 1 and 2).

Although an association with specific receptor types was not investigated, other authors (MacManus et al 1975) have also observed a biphasic mitogenic response to ACh with optima at approximately 5×10^{-5} M and 10^{-8} M. In their work however the low stimulatory concentration was calcium-independent. Perhaps the different strains of rat from which the thymocytes were derived or the higher cell densities they employed could account for this apparently contradictory result. After exhaustive repetitive experimentation we are convinced that low concentrations of ACh activate a muscarinic receptor to initiate calcium-dependent events which lead to mitosis. This view is reinforced by the findings that mitosis induced by either elevated extracellular calcium concentrations or calcium-dependent mitogens (Morgan et al 1975) is invariably blocked by oestradiol as is the mitogenic response to low acetylcholine concentrations (Table 1).

The mitogenic capacity of low concentrations of

Table 2. The effect of antagonists, ions and steroids on catecholamine-induced mitogenesis. Results are the mean percentage of cells in metaphase at 6 h ± s.e.m. derived from 4–26 separate experiments. Significance higher than the basal control ($P < 0.01$) is indicated as *. Phentolamine mesylate and pimozide were obtained from Ciba and Janssen Pharmaceuticals respectively.

	Percentage of cells in metaphase at 6 h				
Treatment	Control	Adrenaline (5×10^{-6} M)	Isoprenaline (10^{-6} M)	Dopamine (10^{-6} M)	Noradrenaline (10^{-12} M)
No additions	3·5 ± 0·1	6·0 ± 0·2*	6·2 ± 0·3*	6·2 ± 0·2*	5·9 ± 0·3*
Propranolol (10^{-6} M)	3·0 ± 0·1	3·0 ± 0·2	3·1 ± 0·1	—	6·0 ± 0·2*
Phentolamine (10^{-6} M)	3·4 ± 0·2	5·9 ± 0·3*	5·4 ± 0·2*	—	3·2 ± 0·2
Pimozide (10^{-6} M)	3·6 ± 0·2	5·8 ± 0·2*	6·4 ± 0 2*	3·8 ± 0·3	—
Calcium omitted	3·6 ± 0·3	5·8 ± 0·2*	6·3 ± 0·2*	5·9 ± 0·4*	3·4 ± 0·1
Magnesium omitted	3·7 ± 0·2	—	3·8 ± 0·2	3·9 ± 0·5	6·1 ± 0·3*
Oestradiol (0·1 μg ml^{-1})	3·7 ± 0·3	5·6 ± 0·3*	7·3 ± 0·6*	5 7 ± 0·4*	3·2 ± 0·2
Testosterone (0·1 μg ml^{-1})	3·5 ± 0·2	—	3·9 ± 0·4	3·8 ± 0·2	6·3 ± 0·4*

ACh and noradrenaline thus links them with a group of calcium-dependent hormonal mitogens which includes insulin and histamine (Morgan et al 1975). In other tissues this calcium-dependency is also associated with an increase in cyclic guanosine monophosphate (cGMP) production (Illiano et al 1973; Kuo et al 1972; Schultz et al 1973; Berridge 1981). Since the exogenous addition or endogenous genesis of this cyclic nucleotide has frequently been invoked as a proliferative stimulant (Friedman 1976; Rebhun 1977; Whitfield et al 1976; Morgan et al 1977; Hunt & Martin 1980) it seemed plausible to suggest an association between cGMP and the proliferation induced by ACh and noradrenaline. Such an association however seems most unlikely. Although ACh can increase cGMP concentrations in thymic lymphocytes, it does so only at non-mitogenic concentrations (MacManus et al 1975 and our own unpublished results). An agonist-induced calcium influx coupled to the regulatory protein calmodulin and thus linked to DNA synthesis and mitosis seems a more likely mechanism at present (Whitfield 1982; MacManus 1982). Indeed, preliminary investigations in this laboratory indicate that calmodulin inhibitors will block calcium-associated mitotic induction whilst leaving magnesium-induced mitogenesis unaffected.

The mitogenic actions of the β-agonists and of dopamine and high concentrations of ACh (Fig. 1, Tables 1, 2) probably are associated with an increase in cyclic adenosine monophosphate (cAMP). In thymic lymphocytes and other tissues these agents can activate adenylate cyclase (Uzunov & Weiss 1972; MacManus et al 1971; Morgan et al 1976; Kebabian & Cote 1981) and a surge of cAMP formation is a prelude to DNA synthesis in a wide variety of cells (Whitfield et al 1980). The obligatory requirement for extracellular magnesium in the mitogenic action of these agents is not easy to explain. Since a high exogenous mitogenic concentration of cAMP itself needs extracellular magnesium to be affective (Morgan et al 1977) it is probably not the binding of agonist to receptor which requires magnesium but some step beyond the genesis of the cyclic nucleotide. It has been suggested that cAMP may promote the influx of magnesium into the cell and it is the increase in intracellular magnesium which initiates DNA synthesis (Perris & Morgan 1976). Adrenaline has been shown to increase magnesium accumulation in adipocytes (Elliott & Rizack 1974) but in lymphoma cells a decrease is apparent (Maguire & Erdos 1980).

The use of the steroids oestradiol and testosterone at rather high concentrations (0·1 μg ml^{-1}) to block calcium-dependent and magnesium dependent mitogenesis is not intended to suggest a physiological role for the steroids in this context. Rather we use them solely to confirm and emphasise the existence of the two discrete mitogenic axes controlled by the two divalent cations. Whether the steroids impair the putative ionic influxes or block some subsequent intracellular event remains to be established.

It is clear that high concentrations of adrenaline, dopamine and ACh are required to initiate the magnesium-dependent events which lead to mitosis suggesting perhaps that the β-adrenergic, dopaminergic and nicotinic receptors on the lymphocyte surface are of low affinity. Whether the high concentrations of agonists required to activate them could be achieved naturally seems unlikely. The much lower concentrations of noradrenaline and ACh needed to activate the α-adrenergic and muscarinic receptors are physiologically more plausible. The responding lymphoblasts must presumably bear only a very small number of high affinity receptors for these compounds upon their surface which when activated trigger calcium-dependent events which lead to mitosis. A realistic possibility for the autonomic control of lymphoid cell proliferation has thus been established.

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