Interleukin-3-stimulated haemopoietic stem cell proliferation

Evidence for activation of protein kinase C and Na^+/H^+ exchange without inositol lipid hydrolysis

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Interleukin 3 (IL-3) is an important regulator of haemopoietic stem cell proliferation both *in vivo* and *in vitro*. Little is known about the possible mechanisms whereby this growth factor acts on stem cells to stimulate cell survival and proliferation. Here we have investigated the role of intracellular pH and the Na^+/H^+ antiport in stem cell proliferation using the multipotential IL-3-dependent stem cell line, FDCP-Mix 1. Evidence is presented that IL-3 can stimulate the activation of an amiloride-sensitive Na^+/H^+ exchange via protein kinase C activation. IL-3-mediated activation of the Na^+/H^+ exchange is not observed in FDCP-Mix 1 cells where protein kinase C levels have been down-modulated by treatment with phorbol esters. Also the protein kinase C inhibitor H7 can inhibit IL-3-mediated increases in intracellular pH. This activation of Na^+/H^+ exchange via protein kinase C has been shown to occur with no measurable effects of IL-3 on inositol lipid hydrolysis or on cytosolic Ca^{2+} levels. Evidence is also presented that this IL-3-stimulated alkalization acts as a signal for cellular proliferation in stem cells.

INTRODUCTION

Interleukin 3 (IL-3) is a multilineage haemopoietic growth factor which can stimulate the survival, proliferation and differentiation of stem cells and a variety of committed progenitor cells *in vitro* (Metcalf, 1984, 1985). IL-3 has also been demonstrated to have stimulatory effects on haemopoiesis *in vivo* (Kindler *et al.*, 1986; Lord *et al.*, 1986), suggesting that this growth factor may be an important physiological regulator of stem cell proliferation and blood cell production. Furthermore, there is evidence to indicate that the abrogation of the dependence of haemopoietic stem and progenitor cells on exogenous growth factors such as IL-3 may be an important factor in leukaemic transformation. For example, the expression of oncogenes such as *myc* (Rapp *et al.*, 1985) or *abl* (Pierce *et al.*, 1985; Cook *et al.*, 1985) can lead to survival and proliferation and malignant transformation of IL-3-dependent cells in the absence of added growth factor with no evidence for autocrine production of IL-3.

The mechanism(s) by which these oncogenes subvert the normal requirement for growth factor is unknown, but is presumably related to the biochemical pathways initiated and maintained by such factors. Thus, an understanding of the mechanisms of receptor-mediated stem cell proliferation is of obvious importance in understanding the regulation of normal cell growth and development and the possible ways in which this can be bypassed leading to leukaemogenesis (see Dexter *et al.*, 1984).

Most of the work performed to elucidate the mechanism of growth factor-stimulated cell proliferation has

been performed on connective tissue cells and little is known of the responses elicited in haemopoietic cells, and in particular of the effects of growth factors on the multipotent stem cells. We have attempted to elucidate some of the biochemical events elicited by IL-3 leading to stem cell survival and proliferation.

Interleukin 3, like other peptide growth factors, mediates its effects in responsive cells by binding to specific high-affinity cell surface receptors (see Walker *et al.*, 1985; Nicola & Peterson, 1986; Sorenson *et al.*, 1986). We have shown that IL-3 binding stimulates the primary metabolism of stem cells leading to maintenance of ATP levels within the cell. Removal of IL-3 leads to a rapid fall in primary metabolism resulting in cell death within 12–48 h (Whetton *et al.*, 1984; Hasthorpe *et al.*, 1987). Studies on the possible transmembrane signalling events governed by IL-3 leading to the maintenance of primary metabolism suggests that IL-3 can translocate and activate a Ca^{2+} -sensitive phospholipid-dependent protein kinase (protein kinase C; PK-C) (Farrar *et al.*, 1985; Whetton *et al.*, 1986a). The tumour-promoting phorbol esters are known to bind and activate PK-C (see Nishizuka, 1984), and as phorbol esters can partially replace the requirement for IL-3 in stem cells it has been inferred that PK-C may have an essential function in IL-3-stimulated cellular proliferation (Whetton *et al.*, 1986a). Additional evidence in support of this notion is that IL-3 and phorbol esters cause phosphorylation of a common set of substrate proteins on threonine and serine residues (Evans *et al.*, 1986). There is also some evidence to suggest that IL-3 receptor occupation activates a tyrosine-specific kinase (Koyasu *et al.*, 1987).

Conventionally, the major route for PK-C activation

Abbreviations used: IL-3, interleukin 3; PK-C, protein kinase C; BCECF, bis(carboxyethyl)carboxyfluorescein; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; 5-MNIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; EGF, epidermal growth factor.

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in response to growth factors is believed to occur by generation of the second messenger diacylglycerol from phosphatidylinositol 4,5-bisphosphate (Nishizuka, 1984; Berridge, 1984; Bell, 1986). When we assessed the effects of IL-3 on phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate within stem cells we found that this growth factor does not stimulate the hydrolysis of these lipids (Whetton *et al.*, 1986b, 1988). This implies that IL-3 activation of PK-C occurs via a mechanism that does not involve phosphoinositide breakdown.

We have extended our studies on the mode of action of IL-3 and present further evidence that IL-3 can activate PK-C without stimulating inositol lipid hydrolysis. Here we demonstrate that IL-3, like many growth factors (e.g. platelet-derived growth factor; see Moolenaar *et al.*, 1983; Rozengurt, 1986; Moolenaar, 1986) can activate Na^+/H^+ exchange via the action of PK-C leading to increases in the pH_i of stem cells. However, unlike other growth factors, IL-3 apparently increases intracellular pH without the concomitant modulation of cytosolic Ca^{2+} levels (Hesketh *et al.*, 1985; Rozengurt, 1986). These data suggest growth factor-mediated control of haemopoietic stem cell proliferation has some distinct differences to the action of other growth factors on their appropriate target cells.

METHODS

Cell culture

FDCP-Mix 1 cells were employed throughout this study. This cell line was derived from long term marrow cultures as described by Spooner *et al.* (1986). The cells have primitive morphology, a diploid karyotype and are non-leukaemic. FDCP-Mix 1 cells are IL-3-dependent for both survival and growth and can self-renew indefinitely in appropriate culture conditions. When exposed to other growth factors or to stromal cells the FDCP-Mix 1 cells can also develop along several distinct haemopoietic lineages (Spooner *et al.*, 1986). These observations show that FDCP-Mix 1 cells have very many characteristics in common with normal, freshly isolated, haemopoietic stem cells, i.e. they represent a self-renewing, multipotent cell which can respond to IL-3 or to marrow stromal cells and undergo multilineage differentiation. FDCP-Mix 1 cells were routinely maintained (in culture conditions promoting self-renewal of the cells rather than differentiation) as described by Spooner *et al.* (1986) and Whetton *et al.* (1986a).

Determination of cellular survival and DNA synthesis

Measurements of cellular viability and DNA synthesis were performed as described by Whetton *et al.* (1986a) except that bicarbonate buffer was replaced by Hepes (25 mM) buffer, pH 7.2.

Determination of [^3H]phorbol dibutyrate binding

Phorbol dibutyrate binding to FDCP-Mix 1 cells was determined as previously described by Whetton *et al.* (1988).

Labelling of FDCP-Mix 1 cells with [^3H]inositol and analysis of [^3H]inositol phosphates

FDCP-Mix 1 cells were incubated in 5% (v/v) horse serum and 5% (v/v) WEHI-3B cell conditioned medium (as a source of IL-3) in Fischers medium. This was

supplemented with 5 μCi of [^3H]inositol/ml. The FDCP-Mix 1 cells were incubated for 18 h at 37 °C with no apparent loss in cellular viability. The cells were then washed and preincubated for 2 h as described below for pH_i determinations prior to challenge with growth factors. After this preincubation the [^3H]inositol-loaded cells were centrifuged, resuspended in phosphate-buffered saline plus 10 mM-glucose to a concentration of $1.3 \times 10^6/\text{ml}$ and placed in 500 μl aliquots. Lithium chloride was added to a final concentration of 10 mM and the cells incubated at 37 °C for 10 min. Appropriate growth factors were added to the cells which were incubated for 10 min. The incubations were terminated, neutralized and prepared for anion exchange chromatography as previously described (Whetton *et al.*, 1986a, 1988).

Determination of intracellular cytosolic Ca^{2+} levels

Measurements of cytosolic Ca^{2+} values ($[\text{Ca}^{2+}]_i$) were made using the Ca^{2+} -sensitive fluorescent probe quin 2 (Tsien *et al.*, 1982). These measurements were made as described in Whetton *et al.* (1988) with the exception that cells were loaded with quin 2 acetoxymethylester at a concentration of 2 μM for 5 min, and all washing and fluorescence determination procedures employed the buffer described below for determination of intracellular pH.

Measurement of intracellular pH

pH_i was determined using the fluorescent pH-sensitive probe bis(carboxyethyl) carboxyfluorescein (BCECF) (Rink *et al.*, 1982). For these experiments cells (unless otherwise stated) were taken from mid exponential-phase cultures and washed twice to remove serum and IL-3. These cells were then resuspended to a concentration of approx. $(2-4) \times 10^6/\text{ml}$ in a buffer consisting of 8.1 mM- Na_2HPO_4 , 1.4 mM- KH_2PO_4 , 2.6 mM-KCl, 136 mM-NaCl and 10 mM-glucose, final pH 7.2 plus 1% (w/v) bovine serum albumin, and incubated for 2 h at 37 °C. After this period the cells were centrifuged, resuspended in the above buffer excluding bovine serum albumin (buffer A) to a concentration of approx. $1 \times 10^7/\text{ml}$. The acetoxymethylester derivative of BCECF was then added to a final concentration of 2 μM and the cells incubated for 10 min at 37 °C. Cells were then washed twice in Buffer A and resuspended in the same buffer for fluorescence determinations. The fluorescence measurements were made in a Perkin-Elmer LS5 fluorescence spectrometer modified to allow constant stirring and maintenance of the contents of the cuvette at 37 °C. Fluorescence from the BCECF-loaded cells was monitored at an excitation wavelength of 500 nm with a 5 nm slit width and emission wavelength of 530 nm, 10 nm slit width. To calibrate the change in fluorescence intensity with respect to pH the cells were lysed with digitonin and the fluorescence recorded at known pH values. Because BCECF apparently behaves differently within the cell compared with the extracellular medium, it was necessary to determine a correction factor to take account of this discrepancy. This correction factor was determined by equilibrating extracellular pH with intracellular pH using nigericin (10 $\mu\text{g}/\text{ml}$) in medium containing 130 mM-KCl to determine fluorescence within FDCP-Mix 1 cells at set pH values. The cells were then lysed and a second calibration curve prepared; the difference between these two curves was found to be a

Fig. 1. Effects of varying doses of IL-3 and TPA on $[^3\text{H}]$ thymidine incorporation and intracellular pH in FDCP-Mix 1 cells

Results shown for intracellular pH experiments are representative of at least three similar experiments. Results of $[^3\text{H}]$ thymidine incorporation experiments are expressed as the mean $\pm$ S.D. of four experiments.

constant 0.15 pH units (between pH 6.5 and 7.4) (Rink *et al.*, 1982).

Interleukin 3

IL-3 was a recombinant product supplied by Biogen. Biological activity (approx. 10^7 units/mg of protein) was determined as described by Bazill *et al.* (1983) using the FDC-P2 cell line.

RESULTS

IL-3 and TPA induce proliferation of FDCP-Mix 1 cells

IL-3 stimulated a dose-dependent increase in $[^3\text{H}]$ -thymidine incorporation in the FDCP-Mix 1 stem cell line (Fig. 1). The phorbol ester TPA (100 ng/ml) in part replaced this requirement for IL-3, leading to survival and proliferation of FDCP-Mix cells (see Fig. 1 and Whetton *et al.*, 1986a). We have previously shown that removal of IL-3 from dependent cell lines results in significant metabolic changes within hours of this occurrence, and that cell death can be irreversible after as little as 8 h without IL-3 (Whetton & Dexter, 1983; Whetton *et al.*, 1984). For this reason cells in the experiments described below were never removed from IL-3 for periods in excess of 4 h prior to use in any experiment.

IL-3 and TPA induce a rapid increase in intracellular pH in FDCP-Mix 1 cells

The addition of IL-3 to FDCP-Mix 1 cells, deprived of the growth factor for 2 h, led to a rapid increase in pH_i as measured by the intracellular pH-sensitive fluorescent probe BCECF (Fig. 1). Addition of IL-3 (100 units/ml) resulted in an increase in pH_i which was first detectable after approx. 2 min, maximal rates of increase occurring about 5 min after addition of the growth factor; the mean increase in pH_i was 0.14 ± 0.03 (mean of six observations $\pm$ S.D.) from a resting pH_i of 7.02 ± 0.03 ($n = 6$, mean $\pm$ S.D.). The extent of the change observed in pH_i was dependent on the dose of IL-3 added to the cells (see Fig. 1). In many other cell types, the phorbol ester TPA can stimulate intracellular alkalization (see Rozengurt, 1986; Moolenaar, 1986). Such a change in pH_i was also observed in FDCP-Mix 1 cells treated with TPA (100 ng/ml). After an initial lag of approx. 1 min there was a rapid increase in pH_i of 0.18 ± 0.04 ($n = 6$, mean $\pm$ S.D.) upon TPA addition (see Fig. 1).

IL-3- and TPA-mediated increases in pH_i are achieved through the activation of an Na^+/H^+ exchanger

Growth factor-mediated increases in intracellular pH are often achieved through the activation of an Na^+/H^+ exchange system at the plasma membrane. TPA-stimulated protein kinase C can activate this Na^+/H^+ exchanger (Rozengurt, 1986; Moolenaar, 1986). The Na^+/H^+ exchanger requires extracellular Na^+ for activity and is inhibited by the diuretic amiloride and its analogues (L'Allemain *et al.*, 1984; Simchowicz & Cragoe, 1986). We have employed these properties to establish whether the IL-3-induced alkalization was achieved via this exchanger. The IL-3- and the TPA-mediated increase in pH_i required the presence of extracellular Na^+ (Fig. 2). Similarly there was a marked inhibition of the IL-3- and TPA-stimulated alkalization

Fig. 2. Effect of 5-MNIA and extracellular Na^+ on IL-3- and TPA-stimulated increases in intracellular pH in FDCP-Mix 1 cells

Results are representative of at least three similar experiments. The final concentrations were 5-MNIA (10 μM), IL-3 (100 units/ml) and TPA (100 ng/ml).

Fig. 3. Effect of down modulation of PK-C on IL-3- and TPA-stimulated increases in intracellular pH and [3 H]thymidine incorporation

Results of pH experiments shown are typical of three such experiments. [3 H]Phorbol dibutyrate and [3 H]thymidine incorporation data are expressed as the means $\pm$ s.d. of three observations, and correspond to the same set of cells for which pHi data is shown. Two other experiments gave similar results. Final concentrations of IL-3 and TPA were 100 units/ml and 100 ng/ml respectively.

Fig. 4. Effect of the PK-C inhibitor H7 on IL-3- and TPA-stimulated increases in intracellular pH

Results shown are representative of three such experiments. IL-3 was present at 100 units/ml and TPA at 100 ng/ml.

by 5-(*N*-methyl-*N*-isobutyl)amiloride (5-MNIA), a potent Na^+/H^+ exchange inhibitor (see Fig. 2). These data suggest that IL-3 stimulates the activation of a Na^+/H^+ exchanger in the FDCP-Mix stem cell line. The bicarbonate-free medium employed for these assays precludes the possibility of an HCO_3^- -linked exchange system leading to intracellular alkalization (L'Allemain *et al.*, 1985).

IL-3 stimulates Na^+/H^+ exchange via an activation of PK-C

To determine whether the observed increases in pHi could be attributed to PK-C activation, two approaches

were taken. Firstly, cells were pretreated over a 16 h period with phorbol dibutyrate (100 ng/ml) as previously described (Whetton *et al.*, 1988). After this period and a further 2 h preincubation in the absence of IL-3 (see under 'Methods'), the FDCP-Mix cells displayed a [3 H]phorbol dibutyrate binding capacity of $15 \pm 4\%$ ($n = 4$, mean $\pm$ s.d.) of control cells. This reduction in [3 H]phorbol dibutyrate binding can be interpreted as a decrease in cellular levels of PK-C (Nishizuka, 1984; Parker & Ullrich, 1986). We measured the effect of IL-3 and TPA on intracellular pH levels in FDCP-Mix cells with depleted PK-C levels and in a control set of cells. The control cells displayed both an IL-3 and TPA-

Fig. 5. Effect of extracellular pH on IL-3-stimulated proliferation in nominally bicarbonate-free Hepes (25 mM)-buffered Fischer's medium

The effect of extracellular pH on [³H]thymidine incorporation at low (0.5 unit/ml; ○) and high (100 units/ml; ●) IL-3 concentrations are shown. Other details of cell culture are as described under 'Methods'.

stimulated alkalization whereas 'down-modulated' cells displayed no such alkalization (Fig. 3), indicating that PK-C mediates IL-3- and phorbol ester-stimulated alkalization of stem cells.

The second approach involved the use of the PK-C inhibitor H7 which has been shown to inhibit this enzyme in many different cell types (see Hidaka & Higiwara, 1987). H7 inhibited TPA-stimulated stem cell alkalization with an ID₅₀ of approx. 50 μM; similarly, IL-3-stimulated alkalization was inhibited with an ID₅₀ of approx. 50 μM (see Fig. 4). The similar ID₅₀ for the H7 inhibition of IL-3- and TPA-mediated alkalization events indicate that IL-3-stimulated increases in pH_i probably involve activation of PK-C.

IL-3-mediated increases in intracellular pH are independent of increased inositol lipid hydrolysis and cytosolic Ca²⁺ fluxes

We have previously shown that IL-3 can stimulate translocation of protein kinase C independently of any increase in inositol lipid hydrolysis or cytosolic Ca²⁺ flux (Whetton *et al.*, 1988). To confirm that IL-3-stimulated increases in pH_i occur with no concomitant increase in these two parameters we have assessed the rate of inositol phosphate formation and cytosolic Ca²⁺ levels under exactly the same conditions as prevail during assays of intracellular pH. Under these conditions IL-3 did not stimulate an increase in [³H]inositol phosphate levels in FDCP-Mix 1 cells, whereas horse serum (employed as a control to demonstrate that these cells were capable of giving such a response; Whetton *et al.*, 1988) increased

these levels by 60%. IL-3 (100 units/ml) had no effect on cytosolic Ca²⁺ levels as assessed by changes in quin 2 fluorescence, whereas horse serum gave a transient increase of approx. 40 nM (results not shown). Also horse serum gave a small increase of 0.03 ± 0.01 pH units (mean ± S.D. from three observations) although this minor effect on pH_i did not persist over longer time periods (see below).

IL-3-mediated activation of protein kinase C and alkalization can influence FDCP-Mix 1 cell proliferation

We have investigated the possible relationship between increased pH_i and stem cell proliferation using several approaches.

(a) In some instances increased extracellular pH can stimulate proliferation (Zetterberg & Engstrom, 1981). To determine whether stem cell proliferation is sensitive to extracellular pH (pH_o) and to establish the optimal pH_o to perform pH_i measurements we have investigated the effect of pH_o on IL-3 mediated survival and proliferation (Fig. 5). At low levels of IL-3 (0.5 unit/ml, sufficient to allow cell survival) pH_o had little effect on [³H]thymidine incorporation; similarly, at high IL-3 doses (100 units/ml) elevated pH_o (> pH 7.2) has an inhibitory effect on IL-3-stimulated [³H]thymidine incorporation whereas cells over a pH_o range from 6.8 to 7.2 gave approximately the same amount of [³H]thymidine incorporation. Cells in the presence of 100 units of IL-3/ml at pH_o values 6.84 and 7.22 had pH_i values of 7.15 ± 0.06 (mean ± S.D., n = 4) and 7.11 ± 0.06 (n = 4) respectively, indicating that pH_o has little effect on pH_i. However, these experiments confirm that the pH 7.20 buffer employed for pH_i determinations was appropriate for FDCP-Mix 1 cells. The increase of extracellular pH to values above 7.3 led to a loss of cellular viability which precluded the possibility of determining meaningful values of pH_i at these pH_o values.

(b) Growth-factor-mediated increases in pH_i are believed to persist for as long as the growth factor is present, or at least for several hours (Moolenaar, 1986). Preincubation of FDCP-Mix 1 cells at low (5 units/ml) and high (100 units/ml) IL-3 concentrations gave a significant difference in [³H]thymidine incorporation levels after 16 h (see Table 1). Assessment of the resting pH_i value of these two sets of cells revealed a difference of 0.15 pH units (see Table 1), the cells held at 100 units of IL-3/ml being the more alkaline. The presence or absence of horse serum (10% v/v) in the overnight incubation had no effect on the pH_i values measured. Furthermore, with no preincubation prior to addition of IL-3 and TPA other than that required for BCECF loading the cells held at low IL-3 concentrations exhibited an acute rise in pH_i on IL-3 addition whereas those held at 100 units of IL-3/ml showed no such increase. Both populations showed a TPA-stimulated alkalization, although this response was somewhat smaller in the cells held at 100 units of IL-3/ml (see Table 1). Furthermore, in cells treated with TPA (100 ng/ml) for 15 min to give a large increase in pH_i ('Methods' as for Fig. 1) there was no increase in pH_i when IL-3 (100 units/ml) was added (results not shown). This is further evidence that these agents may both stimulate cellular alkalization via the common pathway of PK-C activation.

Table 1. Effect of overnight incubation at high and low doses of IL-3 on [³H]thymidine incorporation and intracellular pH

Results shown are the mean $\pm$ S.D. for three observations. Cells were loaded with BCECF as described under 'Methods' except that there was no 2 h preincubation included in the protocol.

Preincubation	[³ H]Thymidine incorporation (c.p.m.)	Resting pH _i	Δ pH _i on addition of	
			TPA (100 ng/ml)	IL-3 (100 units/ml)
IL-3 (5 units/ml)	1892 $\pm$ 178	6.94 $\pm$ 0.03	0.22 $\pm$ 0.04	0.14 $\pm$ 0.02
IL-3 (100 units/ml)	17596 $\pm$ 3381	7.09 $\pm$ 0.02	0.14 $\pm$ 0.03	0.00

(c) Down-modulation of PK-C levels within FDCP-Mix 1 cells inhibited IL-3-stimulated increases in pH_i (Fig. 3). This down-modulation also markedly inhibited IL-3-stimulated proliferation of the stem cells (Whetton *et al.*, 1988; Fig. 3).

(d) The Na⁺/H⁺ exchange inhibitor 5-MNIA markedly inhibited IL-3-mediated [³H]thymidine incorporation over the concentration range where it can also inhibit IL-3-stimulated increases in pH_i (Fig. 6). Maximal effects on IL-3-stimulated alkalization and proliferation were

both achieved with 10 μ M-5-MNIA. The protein kinase C inhibitor H7 can also inhibit DNA synthesis with an ID₅₀ of 50 μ M, similar to that observed for the inhibition of IL-3-stimulated Na⁺/H⁺ exchange.

DISCUSSION

IL-3 can activate PK-C via a novel mechanism

Activation of PK-C is one of the earliest events elicited in stem cells following receptor binding by IL-3 (Whetton *et al.*, 1986a, 1988). Unlike in many other cells this activation of PK-C is not mediated by the hydrolysis of inositol lipids (Whetton *et al.*, 1986b, 1988; Pierce *et al.*, 1988). Here we have shown that IL-3 also stimulates a rapid increase in pH_i in stem cells. That the IL-3-mediated activation of PK-C and the change in pH_i are causally related, as suggested in other systems (Rozengurt, 1986; Moolenaar, 1986) is shown by the observation that TPA (a PK-C activator; Nishizuka, 1984) also increases pH_i in the stem cells and that inhibition or down-modulation of PK-C inhibits IL-3-stimulated pH_i increases (see under 'Results'). Since the changes in pH_i in response to IL-3 are sensitive to 5-MNIA treatment and removal of extracellular sodium ions, this indicates a role for the Na⁺/H⁺ antiport in IL-3-stimulated alkalization. Under conditions where this activation can be shown to occur there is no IL-3-mediated increase in inositol lipid hydrolysis or cytosolic Ca²⁺ levels; nevertheless, the IL-3-stimulated activation of the Na⁺/H⁺ exchange is indeed associated with PK-C activation. This is indicated by our observations that IL-3-stimulated Na⁺/H⁺ exchange is inhibited by the PK-C inhibitor H7 (Fig. 4). Also, when PK-C levels within FDCP-Mix 1 cells are down-modulated to 15% of normal values (Fig. 3) there is no IL-3- or TPA-stimulated increase in pH_i.

Although other growth factors, such as epidermal growth factor (EGF), can stimulate Na⁺/H⁺ exchange with no increase in inositol lipid hydrolysis (Hesketh *et al.*, 1985), the effects of IL-3 on FDCP-Mix 1 cells are somewhat different to those observed in EGF-stimulated Na⁺/H⁺ exchange. For example EGF-mediated increases in pH_i have been shown to be activated by a process independent of PK-C (Vara & Rozengurt, 1985), but this increase in pH_i is accompanied by an increase in cytosolic Ca²⁺ levels (Hesketh *et al.*, 1985). Obviously the actions of IL-3 and EGF differ in some fundamental respect and we are not aware of any other growth factor thus far studied that can activate PK-C without stimulating inositol lipid hydrolysis, with the possible exception of

Fig. 6. Effect of the PK-C inhibitor H7 (■) and Na⁺/H⁺ exchange inhibitor 5-MNIA (□) on IL-3-stimulated [³H]-thymidine incorporation in FDCP-Mix 1 cells

Results shown are the mean $\pm$ S.D. of four observations. IL-3 was present at 100 units/ml.

insulin (Cooper *et al.*, 1987). We are presently investigating the possible mechanisms whereby IL-3 may activate this protein kinase.

IL-3-stimulated Na^+/H^+ exchange is coupled to stem cell proliferation

There is a great deal of evidence to suggest that intracellular alkalization (via activation of Na^+/H^+ exchange) is an important signal in cellular proliferation (see Moolenaar, 1986; Rozengurt, 1986). In agreement with this, we have found that a similar change takes place in stem cells in response to IL-3. Furthermore several lines of evidence indicate that the intracellular pH level of FDCP-Mix 1 cells (as mediated by IL-3) is an important intracellular signal for cellular proliferation.

Firstly, the resting pH_i of cells maintained at low doses of IL-3 (sufficient to allow survival of FDCP-Mix 1 cells without a significant degree of proliferation) and those at high IL-3 levels are markedly different (Table 1). Also, when IL-3-stimulated increases in pH_i are inhibited in three distinct ways, there was a concomitant fall in IL-3-stimulated cellular proliferation. Down-modulation of PK-C leads to reduced IL-3 stimulated DNA synthesis (see Fig. 3). Similarly, the PK-C inhibitor H7 (Figs. 4 and 6) and the Na^+/H^+ exchange inhibitor 5-MNIA (Figs. 2 and 6) both inhibit IL-3 stimulated increases in pH_i and cell proliferation. The PK-C inhibitor H7 will block many protein phosphorylation events that are not associated with Na^+/H^+ exchange activation (see Evans *et al.*, 1986). Nonetheless, the inhibition of Na^+/H^+ exchange and IL-3-stimulated cellular proliferation by H7 and 5-MNIA are consistent with, if not direct evidence for, an association between increased intracellular pH and the mitogenic activity of IL-3.

More positive evidence for this relationship was the observation that TPA can both stimulate [^3H]thymidine incorporation and increase pH_i in FDCP-Mix 1 cells (Fig. 1). However, in PK-C down-modulated cells TPA cannot stimulate either of these two effects of FDCP-Mix 1 cells. Taken together these data indicate that an IL-3-stimulated activation of Na^+/H^+ exchange is an important facet of the pleiotropic responses of stem cells which eventually leads to cell proliferation. It is possible that, as in fibroblasts, increased pH_i is necessary for cellular proliferation but is not a sufficient stimulus to cause proliferation in its own right (Rozengurt, 1986; Moolenaar, 1986).

In summary, we have presented evidence that IL-3 can activate PK-C via a novel mechanism that is independent of inositol lipid hydrolysis. PK-C activation can lead, amongst other events, to the stimulation of Na^+/H^+ exchange and cellular proliferation. Further work on the biochemical basis of the mode of action of this growth factor may lead to some insight into the process by which expression of oncogenes leads to independence from growth factors such as IL-3, and leukaemic transformation.

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