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CSF-1 stimulates Na^+K^+-ATPase mediated $^{86}Rb^+$ uptake in mouse bone marrow-derived macrophages

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$^{86}Rb^+$ was used as an isotopic tracer for the measurement of K^+-uptake into quiescent murine bone marrow-derived macrophages. $^{86}Rb^+$ uptake was inhibited by ouabain indicating a Na^+K^+-ATPase is being measured. In support of this finding, increased sensitivity to ouabain inhibition was seen when the K^+ content of the medium was reduced.

A purified colony stimulating factor (CSF-1) was shown to stimulate the ouabain-sensitive $^{86}Rb^+$ uptake in a dose-dependent manner. Such colony stimulating factor stimulation of $^{86}Rb^+$ (K^+) influx was rapid, with a maximal effect seen 10 minutes after growth factor addition followed by a gradual decrease. Thus increased Na^+K^+-ATPase activity was an early response of macrophages to the colony stimulating factor.

Colony stimulating factor isolated from mouse L-cell conditioned medium (CSF-1) is a two subunit glycoprotein of 70,000 Mr (1,2). This haemopoietic growth factor is specific for cells of the mononuclear phagocytic lineage (3) and mediates its effects on these cells via binding to specific surface receptors (4). CSF-1 is required for the survival, proliferation and differentiation of murine bone marrow-derived macrophages (BMM). Such cells can be prepared as a relatively homogeneous cell population with > 95% of the adherent cells expressing CSF-1 binding (5).

Little is known of the intracellular events involved in the mitogenic signals following binding of CSF-1 to its specific surface receptor. From other cell culture systems there is evidence implicating

Abbreviations:
BMM, bone marrow-derived macrophages; CSF-1, macrophage-specific colony stimulating factor (or macrophage growth factor); DMEM, Dulbecco's modified minimum essential medium; PBS, phosphate buffered saline, pH 7.4.

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the involvement of ion fluxes in the early response to growth factors (6-13). The Na^+K^+-ATPase is a key point in the regulation of such ion redistributions in the cell (14). Other studies have shown that ouabain, a potent inhibitor of the Na^+K^+-ATPase, inhibits myeloid colony formation of progenitor hemopoietic cells (15,16). This indirectly suggests an active involvement of the Na^+K^+-ATPase in hemopoiesis.

We report here that CSF-1 rapidly stimulates a Na^+K^+-ATPase mediated K^+-influx in quiescent BMM. This effect is transient in nature and the degree of stimulation is dependent on the dose of CSF-1.

METHODS

Preparation of bone marrow-derived macrophages

BMM were obtained from precursor cells in bone marrow by the following protocol. Femoral bone marrow cells were isolated from 10 week old male CBA mice (5). Cells were grown in 175 cm^2 Lux tissue culture flasks at $2x10^5$ cells/cm^2 for 3 days in 50 ml of RPMI-1640 supplemented with $5x10^{-5}$M 2-mercaptoethanol, 20mM HEPES, 0.1 g/l neomycin sulfate, 15% heat inactivated fetal bovine serum, 10% L-cell conditioned medium (see below).

For convenience and uniformity, the non-adherent population, containing primitive precursor cells, were collected, washed, and cryogenically preserved in growth medium containing 10% dimethyl sulphoxide and stored in liquid nitrogen. For influx studies these cells were thawed, washed, and seeded into Linbro 12-well dishes at 10^5 cells in 1 ml of growth medium. Adherent BMM were then grown to confluence for 5-6 days. The BMM are a relatively pure and homogeneous population with $\geq$ 95% of the adherent cells binding CSF-1 (5). At this stage cells were washed twice with sterile phospate buffered saline, pH 7.4 (PBS), and recultured in growth medium without L-cell conditioned medium. BMM were generally 'starved' of growth factor for approximately 18 hours prior to use for influx studies. Such factor deprived cells have been shown to be in a quiescent Go/G_1 state of the cell cycle (17,18, unpublished). The protocol typically gave approximately $4x10^5$ adherent cells per well, measured by nuclei counting (19).

K^+-influx

$^{86}Rb^+$ uptake into quiescent BMM was performed either in a HEPES buffer (135mM NaCl,5mM KCl, 0.8mM $MgCl_2$, 1.8mM $CaCl_2$, 5.5mM D-glucose, 50mM HEPES, pH 7.4) or in RPMI-1640 supplemented as described earlier without serum or L-cell conditioned medium and pre-incubated for 30 minutes before further additions as indicated. Na^+K^+-ATPase mediated K^+-influx was measured as ouabain-sensitive $^{86}Rb^+$- uptake. Ouabain (1mM) or buffer was added to the appropriate cultures for 5 minutes prior to pulsing with 0.5 µCi $^{86}Rb^+$ (50 µl). Incubations were in a 37°C shaking water bath for cultures in the HEPES buffer and in a 37°C humidified CO_2 (5% v/v) incubator for cultures in HEPES-bicarbonate buffered RPMI-1640. Uptake was stopped by washing the monolayers 4 times with PBS and the cells solubilized in 1 ml of 0.2M NaOH. Incorporated radioactivity in 0.5 ml of solubilized cells was measured by scintillation counting. Cellular protein was measured using the Coomassie Blue method (20).

Sources of CSF-1

For BMM cultures : Serum containing L-cell conditioned medium for BMM growth was prepared principally as described (21) but with the following

alterations. Mouse L-cells (L-929 strain) were grown in 300 ml Dulbecco's modified minimum essential medium (DMEM) supplemented with 0.1g/l neomycin sulfate, 10% heat inactivated fetal bovine serum in 850 cm^2 Corning plastic roller bottles.

For influx effects : CSF-1 derived from serum-free L-cell conditioned medium was purified to homogeneity as described (22). Briefly the purification stages were: concentrated serum free L-cell conditioned medium (1), calcium phosphate gel absorption (stage 2), DEAE-sepharose chromatography (Stage 3), Ultrogel AcA-44 chromatography (Stage 4), HPLC size exclusion chromatography (Stage 5), HPLC reversed phase chromatography (Stage 6) and rechromatography on reversed phase HPLC (Stage 7). CSF-1 bioactivity was measured using C57Bl bone marrow cells in semi-solid agar medium according to ref 23.

Reagents : The following reagents were obtained commercially: $^{86}RbCl$ (Amersham, Australia), ouabain (Sigma Chemical Company, St. Louis), RPMI-1640, DMEM and fetal bovine serum (Commonwealth Serum Laboratories, Parkville) and Coomassie Blue protein reagent (BioRad Laboratories). All other reagents were of analytical grade. All practical precautions for minimizing endotoxin contamination were taken. Solutions were routinely made up in pyrogen-free water (Abbott Hospital Products) and endotoxin levels were routinely monitored by limulus lysate tests (Commonwealth Serum Laboratories).

RESULTS

$^{86}Rb^+$ has often been used as an isotopic tracer for K^+ (6-13). Fig. 1 shows that for two K^+ concentrations in the medium $^{86}Rb^+$ uptake in BMM is inhibited by ouabain, a specific inhibitor of the Na^+K^+-ATPase

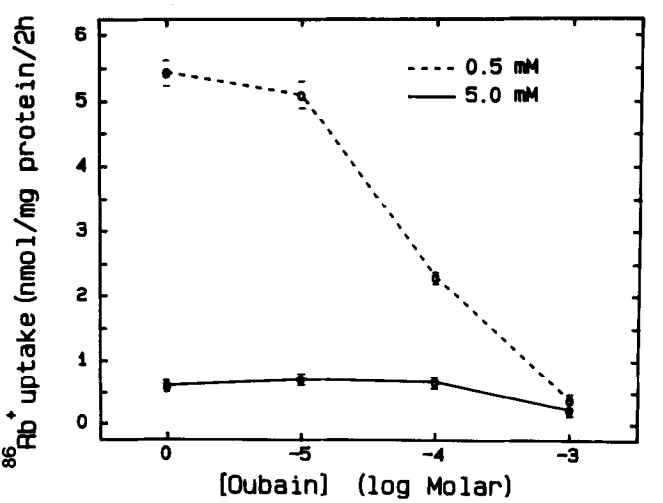

Figure 1: Effect of K^+ concentration on ouabain inhibition of basal $^{86}Rb^+$ uptake in BMM.
Quiescent BMM, prepared as described in Methods, were washed and incubated with HEPES-buffer containing either 5.0mM or 0.5mM K^+. After 30 minutes pre-incubation, the cultures were treated with different concentrations of ouabain and then labelled with $^{86}Rb^+$ for 2 hours. Incorporated $^{86}Rb^+$ was measured as described in Methods. Results represent means of triplicate cultures (± S.E.M.).

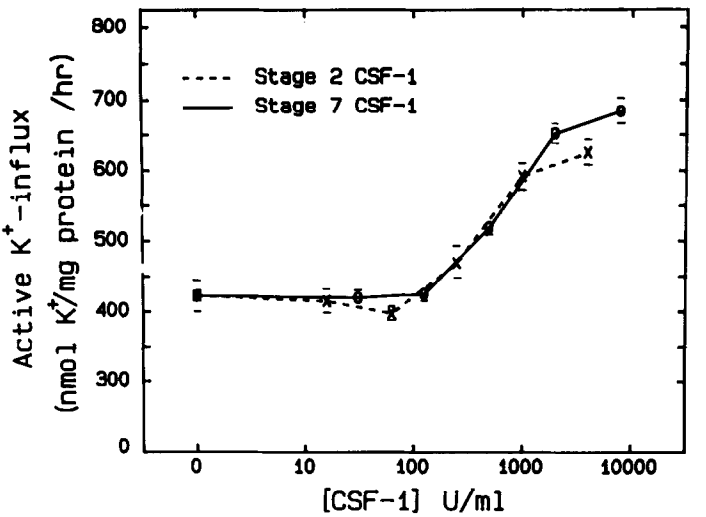

Figure 2: Effect of CSF-1 dose on Na^+ K^+-ATPase activity in BMM. Quiescent BMM were treated with 1mM ouabain in RPMI-1640 or with RPMI-1640 alone and cultured a further 5 minutes. Various doses of pure (stage 7) CSF-1 and partially purified (stage 2) CSF-1 were added simultaneously with $^{86}Rb^+$ for 1 hour. The active K^+-influx represents the difference between ouabain-free and ouabain-treated groups. CSF-1 dilutions were performed in RPMI-1640 in the presence of Tween 20 (0.02% v/v) to minimize loss due to non-specific binding to plastic. Control cultures (no CSF-1) were treated with RPMI-1640 and Tween 20. The final concentration of Tween-20 in the cultures was 0.002%. In the presence of ouabain the value for K^+-influx was approximately 150 nmole K^+/mg cell protein/hr and remained relatively constant for all CSF-1 doses for both CSF-1 preparations. Each point represents the mean of triplicate cultures (± S.E.M.).

(24). A marked increase in sensitivity to ouabain is seen when the K^+ content of the medium is reduced. In 0.5mM K^+ there is a more dramatic effect of 10^{-4}M and 10^{-3}M ouabain compared to these ouabain concentrations in the presence of 5.0mM K^+. Such competition between ouabain and K^+ is a known property of the ouabain inhibition of the Na^+K^+-ATPase (6,24). Fig. 1 also shows that in the absence of ouabain a 10-fold decrease in K^+ content in the medium results in an inversely proportional increase in $^{86}Rb^+$ incorporation into the cells. This indicates that $^{86}Rb^+$ and K^+ are directly competing for transport by the pump and is in accordance with the results obtained in other systems showing $^{86}Rb^+$ as a valid analogue for K^+ for influx studies (6,12,24).

We then examined to examine the effect of CSF-1 on Na^+K^+-ATPase activity. Fig.2 shows that both partially purified (stage 2) and pure

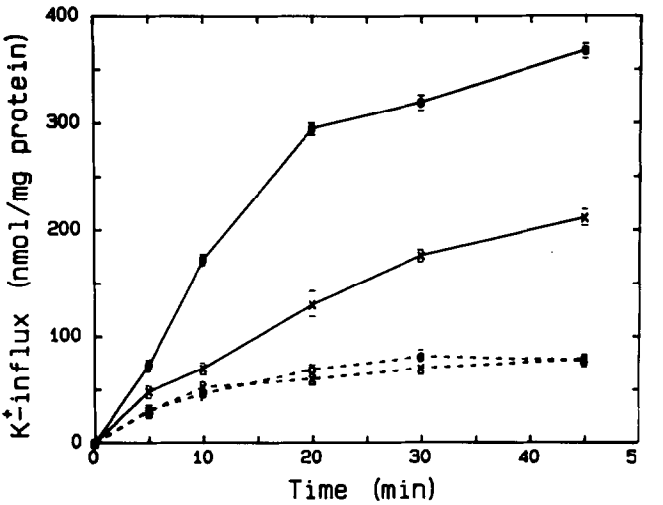

Figure 3: Time course for the stimulation of BMM Na^+ K^+-ATPase activity by CSF-1. Quiescent BMM cells were pre-incubated in HEPES-buffer containing 5mM K^+. After 30 minutes, appropriate cultures were treated with 1mM ouabain or buffer alone and incubated a further 5 minutes. A partially purified CSF-1 preparation (stage 3, 5000 U/ml) was added simultaneously with $^{86}Rb^+$ (0.5 μ Ci/well). Accumulation of $^{86}Rb^+$ was measured at intervals over the next 45 minutes. (x——x), no CSF-1; (o——o), CSF-1; (x---x), oubain; (o---o), CSF-1 + oubain. Each point represents the mean of triplicate cultures (± S.E.M.).

(stage 7) CSF-1 stimulate ouabain-sensitive K^+-influx in a dose dependent manner. Comparison of the dose response curves shows that there is little difference in the ability of the two CSF-1 preparations to stimulate K^+-influx. Stimulation of K^+-influx for both CSF-1 preparations was entirely ouabain sensitive (fig.2). These data show that CSF-1 can stimulate the Na^+K^+-ATPase mediated K^+-influx. For the remaining experiments partially purified CSF-1 was used.

The data in fig.3 illustrate the total incorporation of K^+ into quiescent BMM over a period of 45 minutes in the presence and absence of CSF-1. Addition of CSF-1 results in a significant increase in K^+-influx, seen within 5 minutes after growth factor addition. Ouabain completely inhibits any CSF-1 stimulation of K^+-influx. Since fig.3 indicated a distinct temporal enhancement of K^+-influx by CSF-1 a more detailed kinetic analysis was performed.

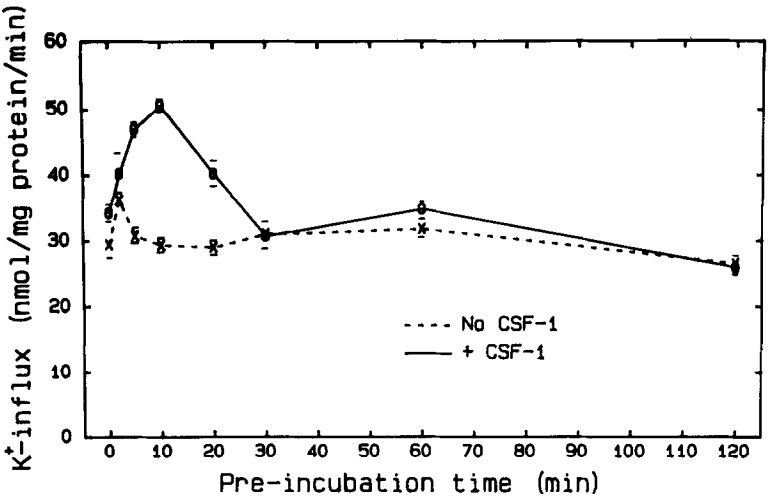

Figure 4: Kinetics of the stimulation of BMM Na^+ K^+-ATPase activity by CSF-1. Quiescent BMM cells were washed and pre-incubated for 30 minutes in HEPES-bicarbonate buffered RPMI-1640. Cells were pulsed for 1 minute with 0.5 μ C_i $^{86}Rb^+$ per well (1ml final volume) at varying intervals after the addition of a partially purified CSF-1 preparation (stage 4, 5000 U/ml) or RPMI alone. Each point represents the mean of triplicate cultures ± S.E.M.

Fig. 4 shows the time course of stimulation of the Na^+ K^+-ATPase by CSF-1. Quiescent BMM were pulsed for 1 minute at varying intervals after the addition of partially purified CSF-1. There is a significant increase in the rate of K^+-influx (Na^+ K^+-ATPase activity) occurring within 1 minute after growth factor addition, rising sharply up to 10 minutes, at which point the rate decreased gradually. In several experiments a return to a slightly enhanced level above that for the untreated group was found for the CSF-1 stimulated cells.

DISCUSSION

The present results indicate that $^{86}Rb^+$ uptake in BMM is inhibited by ouabain, with the sensitivity to inhibition being dependent on the K^+ content of the medium. There is also direct competition between $^{86}Rb^+$ uptake and the K^+ content of the medium. These are properties common to Na^+ K^+-ATPase mediated K^+-transport found in other cellular systems (6,12,24) and it suggests that $^{86}Rb^+$, as a K^+ analogue, is being transported in a similar manner in the BMM system.

Addition of partially purified CSF-1 preparations to quiescent BMM monolayers results in a dose-dependent stimulation of ouabain-sensitive $^{86}Rb^{+}$ uptake. Pure CSF-1 results in the same dose response curve and therefore indicates that the CSF-1 molecule is the active component in the partially purified preparations. There is no effect on the ouabain-insensitive component of $^{86}Rb^{+}$ uptake suggesting that CSF-1 is mediating its effect on K^{+}-influx solely via the Na^{+} K^{+}-ATPase.

Stimulation of Na^{+} K^{+} -ATPase mediated K^{+}-uptake by CSF-1 changes with time after addition of the growth factor. Quiescent BMM when treated with CSF-1 show a rapid increase in Na^{+} K^{+}-ATPase activity detected within 1 minute after CSF-1 addition rising sharply to a maximum level at 10 minutes, then falling off gradually. Such transient increases in K^{+}-influx have also been seen in other cell types treated with various growth promoting agents (6,7,9,11).

The Na^{+} K^{+}-ATPase, via its influence on the absolute levels and gradients of Na^{+} and K^{+}, and also on the ATP/ADP ratio within the cell, can have an effect on a variety of diverse cellular processes. These include nutrient transport (e.g. sugar and amino-acids), transport of other ions (H^{+}, Ca^{2+}, Mg^{2+}), and activity of a variety of cellular enzymes (14). Recently it has been shown that CSF-1 stimulates protein synthesis and inhibits protein degradation in BMM, and that the relative rates of these two distinct processes play an important part in the pleiotropic response mediated by CSF-1 (25). It was not determined how CSF-1 mediated this effect. In view of the known relationship between intracellular K^{+} levels and protein synthetic rate (26), it is interesting to speculate that CSF-1 stimulation of Na^{+} K^{+}-ATPase may be an important mechanism by which CSF-1 stimulates protein synthesis and thereby regulates macrophage proliferation. In view of the rapid response of the Na^{+} K^{+}-ATPase to CSF-1 and the importance of this membrane bound enzyme in regulating diverse cellular processes, further

investigation into any cause-effect relationship between Na^+ K^+-ATPase activity and DNA synthesis is warranted.

ACKNOWLEDGEMENTS

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