Amiloride added together with bumetanide completely blocks mouse 3T3-cell exit from G0/G1-phase and entry into S-phase

Rivka PANET,* David SNYDER and Henri ATLAN

Department of Medical Biophysics and Nuclear Medicine, Hadassah University Hospital, P.O.B. 12000, 91120 Jerusalem, Israel

In this study we tested the hypothesis that stimulation of univalent-cation fluxes which follow the addition of growth factors are required for cell transition through the G1-phase of the cell cycle. The effect of two drugs, amiloride and bumetanide, were tested on exit of BALB/c 3T3 cells from G0/G1-phase and entry into S-phase (DNA synthesis). Amiloride, an inhibitor of the Na^+/H^+ antiport, only partially inhibited DNA synthesis induced by serum. Bumetanide, an inhibitor of the Na^+/K^+ co-transport, only slightly suppressed DNA synthesis by itself, but when added together with amiloride completely blocked cell transition through G1 and entry into S-phase. Similar inhibitory effects of the two drugs were found on the induction of ornithine decarboxylase (ODC) (a marker of mid-G1-phase) in synchronized cells stimulated by either partially purified fibroblast growth factor (FGF) or serum. To test this hypothesis further, cells arrested in G0/G1 were stimulated by serum, insulin or FGF. All induced similar elevations of cellular K^+ content during the early G1-phase of the cell cycle. However, serum and FGF, but not insulin, released the cells from the G0/G1 arrest, as measured by ODC enzyme induction. This result implies that the increase in cellular K^+ content may be necessary but not sufficient for induction of early events during the G1-phase. The synergistic inhibitory effects of amiloride and bumetanide on the two activities stimulated by serum growth factors, namely ODC induction (mid-G1) and thymidine incorporation into DNA (S-phase), suggested that the amiloride-sensitive Na^+/H^+ antiport system together with the bumetanide-sensitive Na^+/K^+ transporter play a role in the mitogenic signal.

INTRODUCTION

One of the earliest responses of quiescent cells to a mitogenic signal is activation of the Na^+/H^+ exchange (Smith & Rozengurt, 1978; Koch & Leffert, 1979; Villereal, 1981; Moolenaar *et al.*, 1982; Schuldiner & Rozengurt, 1982; Pouyssegur *et al.*, 1982; Boonstra *et al.*, 1983; Frelin *et al.*, 1983). It was shown that this increased Na^+ influx stimulated the Na^+/K^+ -pump activity (Smith & Rozengurt, 1978; Rozengurt & Mendoza, 1980) and the subsequent increase in cellular K^+ (Tupper *et al.*, 1977; Mendoza *et al.*, 1980; Frantz *et al.*, 1981; Lopez-Rivas *et al.*, 1982). It has been proposed that the increase in intracellular K^+ and Na^+ , and the elevation of the pH induced by growth factors at the early G1-phase of the cell cycle, are part of the signal for proliferation (Schuldiner & Rozengurt, 1982; L'Allemain *et al.*, 1984). Further evidence, however, indicated that rapid stimulation of the Na^+/H^+ exchange system is necessary, but not sufficient, for the induction of cell proliferation. For example, several agents caused cytoplasmic alkalization by stimulation of the Na^+/H^+ antiport without stimulation of mitogenesis (Rothenberg *et al.*, 1983; Besterman *et al.*, 1984). Amiloride and amiloride analogues under certain experimental conditions (in the presence of $\text{CO}_2/\text{HCO}_3^-$) completely inhibited the Na^+/H^+ exchange activity, but failed to inhibit stimulation of DNA synthesis by growth factors (L'Allemain *et al.*, 1984).

Two other Na^+ -transport systems were shown to be stimulated in quiescent cells by growth factors: the

ouabain-sensitive Na^+/K^+ pump (Rozengurt & Heppel, 1975; Smith, 1977; Tupper *et al.*, 1977; Panet *et al.*, 1982, 1983) and the 'loop' diuretic-sensitive Na^+/K^+ transporter (bumetanide-sensitive transporter) (Tupper *et al.*, 1977; Panet *et al.*, 1982, 1983; Panet, 1985; Amsler *et al.*, 1985). These systems, which transport both K^+ and Na^+ , were shown to be responsible for the elevated cellular K^+ content at the early G1-phase (Tupper *et al.*, 1977; Panet, 1985). The question of whether the increase in cellular pH, Ca^{2+} , Na^+ or K^+ content induced by growth factors at early G1-phase is the signal for proliferation has not been solved. The present study was undertaken to elucidate the ionic events essential for cell release from G0/G1 phase by the growth factors. The effect of two inhibitors of the univalent-cation transporters was measured on cell response to growth factors.

MATERIALS AND METHODS

[^{14}C]Ornithine was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [^3H]Thymidine was purchased from the Nuclear Research Centre, Beer-Sheva, Israel. Amiloride hydrochloride was kindly provided by Merck, Sharp and Dohme Research Laboratories, West Point, PA, U.S.A. Bumetanide was kindly provided by Laboratoire Leo, 9-28500 Vernouillet, Denmark. Fibroblast growth factor (FGF) was partially purified from bovine brain as described by Gospodarowicz *et al.* (1978), by using CM-Sephadex and one Sephadex G-75 column, which gave 200–300 units/mg (a unit is defined as the amount of growth factor

Abbreviations used: FGF, fibroblast growth factor; ODC, ornithine decarboxylase.

* To whom reprint requests and correspondence should be addressed.

required to yield half-maximal [^3H]thymidine incorporation by 3T3 cells). The FGF was provided by Dr. Israel Vlodavski, Hadassah University Hospital, Jerusalem, Israel. Insulin was purchased from Eli Lilly and Co., Indianapolis, IN, U.S.A.

BALB/c 3T3 fibroblasts were maintained in minimal essential medium containing 5% fetal-calf serum at 37 °C in a humidified atmosphere of air/CO₂ (19:1). Quiescent cultures were obtained by serum deprivation as described previously (Panet *et al.*, 1982). ODC enzyme activity was measured as described by Don & Bachrach (1975). The activity was determined in triplicate plates; it was linear with time for 1 h under the experimental conditions, and was corrected for protein content. Cellular K⁺ content was determined after washing the plates three times with cold MgCl₂ (125 mM) and once with cold NaCl (165 mM). The cells were lysed with 1.0 ml of NaOH (0.1 M), and K⁺ was measured with a Perkin-Elmer atomic-absorption spectrophotometer (Atlan *et al.*, 1984).

RESULTS

Effects of amiloride and bumetanide on cell exit from G0/G1-phase and entry into S-phase

In order to evaluate the role of the two univalent-cation transporters, stimulated by growth factors, we studied the effect of their inhibitors on cell progression through the G1-phase and entry into S-phase. As a mid-G1-phase marker we followed the induction of ODC enzyme 2–6 h after release (Haddox *et al.*, 1980). Partially purified FGF induced cell exit from arrest in G0/G1, as measured by induction of ODC enzyme activity. ODC induction was partially blocked in the presence of 0.1 mM-bumetanide, and more strongly inhibited by 1 mM-bumetanide (Fig. 1). A similar inhibitory effect on ODC induction was found with furosemide, another inhibitor of the Na⁺/K⁺ co-transporter (results not shown). Amiloride (1 mM) strongly but not completely inhibited ODC induction by FGF (Fig. 1). To study the combined effect of the two inhibitors on cell exit from G0/G1-phase of the cell cycle, quiescent cells were

Fig. 1. Effect of amiloride and bumetanide on ODC induction, in cells released by FGF

G0/G1-phase-arrested cells were released by adding fresh medium containing FGF (1 µg/ml) to control cells (○) or to cells in the presence of bumetanide (0.1 mM) (△), bumetanide (1 mM) (▲) or amiloride (1 mM) (□). ODC induction was measured as described in the Materials and methods section.

stimulated by FGF in the presence of increasing amiloride concentrations, with or without bumetanide (0.1 mM) (Fig. 2). Increasing amiloride concentration decreased ODC induction in a dose-dependent fashion. Bumetanide at 0.1 mM only slightly inhibited ODC induction. However, when bumetanide (0.1 mM) was added with increasing concentrations of amiloride, ODC induction was strongly inhibited, until 99% inhibition was observed with 1 mM-amiloride together with 0.1 mM-bumetanide (Fig. 2).

These data can be interpreted in two ways: (1) the two drugs inhibit events during the early G1 phase of the cell cycle; (2) the drugs inhibit ODC induction through a non-specific toxic effect. To differentiate between these possibilities, quiescent cells were stimulated with serum and amiloride was subsequently added at different times. ODC induction was always measured 1–5 h after serum addition (Fig. 3). Amiloride strongly inhibited ODC induction when added within the first 2 h of serum stimulation. However, if amiloride was added 2 h after the cells were released by serum, ODC induction was only slightly inhibited. Similar results were obtained when the cells were released with FGF, and also when bumetanide (0.1 mM) was added together with amiloride (results not shown). This result supports the proposal that amiloride specifically inhibits early events during cell exit from the G0/G1-phase arrest. These findings argue against a toxic effect of amiloride at 1.0 mM concentration; if the amiloride effect were non-specific [for instance, by blocking total protein synthesis (Lubin *et al.*, 1982)], one would expect to observe similar effects on ODC induction at any time after release. The findings that the maximal effect of the two inhibitors is during the first 2 h of serum or FGF addition is in agreement with the proposal that the ionic changes are crucial mainly during the first few hours in the G1-phase (Rozenfurt & Mendoza, 1980). However, one could not exclude the possibility that the effect of these drugs is through

Fig. 2. Combined effect of amiloride and bumetanide on ODC induction by FGF

G0/G1-phase-arrested cells were released by FGF (1 µg/ml) in the absence or presence of the indicated inhibitors. After 4 h, ODC induction was measured as described in the Materials and methods section.

Fig. 3. Effect of amiloride, added at different times after serum stimulation, on ODC induction

G0/G1-phase-arrested cells were released by the addition of 5% fetal-calf serum (zero time), in the absence (○) or presence of 1 mM-amiloride. Amiloride was added at zero time (■), or at 1 h (▲) or 2 h (●) after serum addition. ODC induction was measured at the indicated time as described in the Materials and methods section.

non-specific inhibition of other activities in early G1-phase (Zhuang *et al.*, 1984).

We further investigated the combined effect of the two drugs on cell transition into S-phase of the cell cycle (DNA synthesis) (Fig. 4). Increasing amiloride concentrations (up to 1 mM) inhibited thymidine incorporation into DNA by 35%; on the other hand, bumetanide (1 mM) inhibited DNA synthesis by only 14%. However,

Fig. 4. Effect of amiloride and bumetanide on DNA synthesis in synchronized cultures released by serum

G0/G1 quiescent cells were released by 5% fetal-calf serum for 18 h, and pulse-labelled for 2 h with [³H]thymidine (1 μCi/ml). Trichloroacetic acid-insoluble radioactivity was determined, and results (±S.E.M.) were corrected for protein content of the cell.

when bumetanide (0.1 mM) and amiloride (1.0 mM) were added together at the time of serum stimulation, a complete inhibition (99%) of thymidine incorporation into DNA was observed. Thus the synergistic inhibitory effect of the two drugs on DNA synthesis was observed at high amiloride concentration. The finding that no synergism between the two inhibitors occurs when 0.1 mM-amiloride is used could be due to instability of amiloride incubated for 20–24 h with the cells. In repeated experiments we observed that ODC induction is more sensitive to amiloride inhibition than DNA synthesis is (as shown in Figs. 2 and 4). ODC is considered a marker for mid-G1-phase of the cell cycle (Haddox *et al.*, 1980); however, there may not be an exact correlation between the extent of ODC induction (measured 4 h after release) and DNA synthesis, which occurs 16–20 h later. In addition, amiloride instability could also contribute to the different degree of inhibition, since DNA synthesis was measured 16–20 h after ODC induction.

To test whether the inhibitory effect on DNA synthesis of these drugs was due to a toxic effect, we washed cell cultures which had been incubated for 18 h with serum and the different drugs (black bars, Fig. 5), and fed them with fresh medium containing 5% fetal-calf serum. Then 18 h later we measured cell proliferation by thymidine incorporation into DNA (white bars, Fig. 5). The inhibitory effect of amiloride and bumetanide was reversible; washing the cells from the drugs completely released the cells from the block of DNA synthesis.

Effects of bumetanide and amiloride on cellular K⁺ content increase induced by FGF

The increase in cellular K⁺ content during the early G1-phase of the cell cycle was shown to be a result of

Fig. 5. Reversible inhibition of DNA synthesis by amiloride, bumetanide and furosemide in synchronized cultures

G0/G1-phase-arrested cells were released by the addition of fetal-calf serum (5%) in the presence of the indicated drugs (each at 1 mM concentration). After 18 h, the cells were labelled for 2 h with [³H]thymidine (1 μCi/ml) (black bars). In parallel, other cultures were similarly released in the presence of the drugs for 18 h, then washed and fed with fresh medium containing 5% fetal-calf serum. After a further 18 h these cultures were labelled for 2 h with [³H]thymidine (1 μCi/ml) (white bars). Results of three experiments in triplicate cultures were pooled and expressed as percentage of the control, ±S.E.M.

uphill uptake of K^+ by both the Na^+/K^+ pump (Rozengurt & Heppel, 1975; Tupper *et al.*, 1977; Smith & Rozengurt, 1978; Panet *et al.*, 1982, 1983) and the loop diuretic-sensitive transporter (Tupper *et al.*, 1977; Panet *et al.*, 1982, 1983; Panet, 1985). These two transporter activities are rapidly stimulated after the addition of serum growth factors. Na^+/K^+ -pump stimulation was shown to be the result of increased Na^+ influx through the Na^+/H^+ antiport (Rozengurt & Mendoza, 1980). The finding that the combination of amiloride and bumetanide completely blocked ODC induction suggested that there is a correlation between their effects on cellular Na^+ and as a result on cellular K^+ (amiloride by blocking Na^+/K^+ pump stimulation, and bumetanide by blocking bumetanide-sensitive Na^+/K^+ co-transport) and the inhibitory effect on cell transition through the G1 phase. To study the relationship between cellular K^+ content and cell exit from G0/G1 phase, quiescent cells were stimulated by FGF in the presence of amiloride and bumetanide (Table 1). Amiloride or bumetanide when added separately only partially

Table 1. Effect of amiloride and bumetanide on ODC induction and cellular K^+ content in quiescent cells released by FGF

Quiescent cells were released by the addition of FGF (1 μ g/ml) in the absence or presence of bumetanide (0.1 mM) and amiloride (1 mM). After 5 h ODC activity and cellular K^+ content were measured as described in the Materials and methods section.

Addition	ODC activity (pmol/h per mg)	Cellular K^+ content (μ mol/mg)
—	0	$0.852 \pm 0.051^*$
FGF	2014 ± 144	1.178 ± 0.053
FGF + bumetanide	1486 ± 150	1.128 ± 0.042
FGF + amiloride	371 ± 50	$1.079 \pm 0.040^*$
FGF + bumetanide + amiloride	0	$0.909 \pm 0.050^*$

* These values are significantly different ($P < 0.05$) from the K^+ content with FGF alone as assessed by a *t* test for the difference between the two means.

Fig. 6. Effect of FGF, insulin and fetal-calf serum on ODC induction and K^+ content

G0/G1-phase-arrested cells were released by the addition of fetal-calf serum (5%) (□), FGF (1 μ g/ml) (●) or insulin (10 μ g/ml) (△). ODC enzyme activity and cellular K^+ content were measured as described in the Materials and methods section.

inhibited cellular K^+ increase. Combination of the two drugs completely inhibited the cellular K^+ increase induced by FGF. Similar results were obtained by releasing the cells with serum (results not shown).

Effects of serum, FGF and insulin on ODC induction and cellular K^+ content

To evaluate further the role of cellular Na^+ and K^+ contents after the addition of growth factors to quiescent cells, kinetics of ODC induction and elevation of K^+ content were compared. Releasing quiescent cells by each of the three factors, serum, FGF or insulin, was followed by an increased cellular K^+ content. The increase in intracellular K^+ content by either FGF or insulin was the result of K^+ -influx stimulation (Panet *et al.*, 1986). ODC was induced by serum and FGF only; insulin did not induce ODC activity in the quiescent cells (Fig. 6). This observation suggested that the increased cellular K^+ content (as a result of Na^+ influx increase) stimulated by insulin was not sufficient for the induction of ODC and DNA synthesis (results not shown).

DISCUSSION

Changes in transmembrane fluxes of univalent ions have been implicated in the regulation of the proliferative response mediated by growth factors. It has been proposed that a rapid increase in Na^+ entry stimulates the activity of the Na^+/K^+ pump and increases intracellular K^+ (Rozengurt & Mendoza, 1980; Burns & Rozengurt, 1984). According to this theory, growth factors trigger a ' Na^+ cycle' in which Na^+ influx via an amiloride-sensitive Na^+/H^+ antiport results in pH modulation, followed by Na^+ efflux through the Na^+/K^+ pump; this in turn leads to K^+ accumulation.

We chose to study the participation of univalent ions in the mitogenic signal by examining the effect of inhibitors of the univalent-cation transporters on cell exit from the G0/G1-phase arrest and entry into S-phase. We have used two specific inhibitors, amiloride and bumetanide, each of which inhibits a different transporter

shown to be stimulated by growth factors (Tupper *et al.*, 1977; Smith & Rozengurt, 1978; Koch & Leffert, 1979; Villereal, 1981; Moolenaar *et al.*, 1982; Schuldiner & Rozengurt, 1982; Pouyssegur *et al.*, 1982; Frelin *et al.*, 1983; Panet *et al.*, 1982, 1983; Panet, 1985; Panet *et al.*, 1986). Amiloride, when added separately, only partially inhibits DNA synthesis, which is in accord with previous reports (Besterman *et al.*, 1984; L'Allemain *et al.*, 1984; Amsler *et al.*, 1985).

Further evidence has indicated that the rapid stimulation of the Na^+/H^+ exchange is not a sufficient signal for the induction of cell proliferation (Rothenberg *et al.*, 1983; Besterman *et al.*, 1984; L'Allemain *et al.*, 1984). It has therefore been proposed that the Na^+/H^+ antiport plays a secondary role in the mitogenic signal (L'Allemain *et al.*, 1984). The results in the present paper suggest that the amiloride-sensitive Na^+/H^+ antiport and the bumetanide-sensitive Na^+/K^+ transporter carry out ion fluxes which are essential for cell transition through the G1-phase. This conclusion is based on the observation that, when added individually, the inhibitors only partially inhibited cell entry into S-phase (Figs. 4 and 5). However, when both the Na^+/H^+ antiport and the Na^+/K^+ co-transport are blocked during stimulation with growth factors, complete inhibition of cell entry into S-phase is achieved. It is therefore possible that, if one of these transporters is blocked, the other can compensate, and as a result only partial inhibition of DNA synthesis is obtained.

The possibility that the effects of the two inhibitors on cell transition through the G1-phase is due to a toxic effect (Lubin *et al.*, 1982) should also be considered. In fact, total protein synthesis was inhibited by 50% by 1.0 mM-amiloride, which blocked 95% of ODC induction (Figs. 1–3). No further inhibition of total protein synthesis was observed on the addition of bumetanide (0.1 mM) together with amiloride (results not shown). We have performed the following experiments to address this possibility. First, we studied the effect of amiloride added at different times after release on ODC induction by serum (Fig. 3). If amiloride affected ODC induction mainly through a toxic effect, it should inhibit ODC induction at any time after serum stimulation. We found that it blocked ODC induction only within the first 2 h after release. Secondly, we washed cells that had been treated for 18 h with the two drugs, fed them with fresh medium containing 5% fetal-calf serum, and measured DNA synthesis 18 h after the drugs were washed out (Fig. 5). The inhibitory effect of the two drugs, when added separately or together, was found to be reversible.

The result presented here may support the hypothesis that the synergistic inhibitory effect of amiloride and bumetanide on cell exit from G1-phase arrest and entry into S-phase is through its effect on ionic events stimulated at the early G1-phase. However, amiloride was shown to inhibit other activities in the early G1-phase (Zhuang *et al.*, 1984), in addition to its effect on the Na^+/H^+ antiport. To investigate whether the inhibitory effect of the two drugs is mainly through their inhibition of the ionic events stimulated by serum growth factors, or through other non-specific events, we measured their effect on cellular K^+ increase at the early G1-phase. The synergistic inhibitory effect of the two drugs on cellular K^+ increase may support the hypothesis that the effect of the two drugs is mainly through the inhibition of Na^+ influx into the cell stimulated by serum growth factors:

amiloride, by blocking the Na^+/H^+ antiport, and bumetanide, by blocking $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport. The blockage of the two Na^+ channels would inhibit the increase in cellular K^+ content. However, one could not rule out the possibility that part of the inhibitory effect of the two drugs is due to their effect on other activities stimulated in early G1-phase by serum growth factors. The 35% inhibition of DNA synthesis by 1.0 mM-amiloride could be a result of non-specific effects, which could perhaps be avoided by using amiloride analogues (L'Allemain *et al.*, 1984). We have found that the non-specific effect of amiloride, as measured by protein-synthesis inhibition, does not increase on the addition of bumetanide together with amiloride (results not shown), whereas DNA synthesis was completely blocked (Fig. 4). The possibility that another transporter such as the $\text{Cl}^-/\text{HCO}_3^-$ exchange system may play a role in the mitogenic signal is rather exciting, especially since it was shown to be Na^+ -dependent (L'Allemain *et al.*, 1985). However, in contrast with the Na^+/H^+ exchange, that exchange system does not appear to be activated upon growth-factor addition to quiescent cells (L'Allemain *et al.*, 1985).

Is the elevation in cellular K^+ and/or Na^+ content essential for cell transition through the G1 phase?

The hypothesis that the increase in cellular Na^+ or K^+ content (as a result of increased Na^+ influx) during early G1-phase is essential for the growth-factor-mediated proliferative response is still controversial. Our finding that amiloride and bumetanide when added separately only partially suppressed cellular K^+ increase and ODC induction, whereas the combined effect of the two drugs was total inhibition of both K^+ increase and cell exit from the G0/G1 arrest, favours the above hypothesis. We previously showed that release of mouse 3T3 cells transformed by a temperature-sensitive mutant of Rous sarcoma virus, in the absence of growth factors, is accompanied by an increase in cellular content of K^+ (Panet *et al.*, 1985). This result is in accord with the hypothesis that the increase in cellular K^+ (and Na^+) is an obligatory event for proliferation. However, some evidence in the present work qualifies this conclusion. (1) Insulin was shown to increase K^+ influxes (Rozengurt & Heppel, 1975; Amsler *et al.*, 1985) and as a result to cause elevation of cellular K^+ content (Fig. 6), similar to the K^+ increase obtained by serum growth factors or by FGF. However, in contrast with FGF and serum, insulin did not induce ODC enzyme activity in the arrested cells. (2) Ouabain at concentrations which inhibited the elevation of cellular K^+ content or even caused some depletion of cellular K^+ only partially inhibited ODC induction (results not shown). These considerations taken together would argue that the increase in cellular K^+ (or Na^+) content at the early G1-phase of the cell cycle may be essential, but not sufficient, for proliferation.

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