Stimulation of Bumetanide-sensitive Na+/K+/Cl− Cotransport by Different Mitogens in Synchronized Human Skin Fibroblasts Is Essential for Cell Proliferation

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Abstract. In this study, we examined the role of the bumetanide-sensitive Na+/K+/Cl− cotransport in the mitogenic signal of human skin fibroblast proliferation. The Na+/K+/Cl− cotransport was dramatically stimulated by either fetal calf serum, or by recombinant growth factors, added to quiescent Go/G1 human skin fibroblasts. The following mitogens, FGF, PDGF, α-thrombin, insulin-like growth factor-1, transforming growth factor-α, and the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate, all stimulated the Na+/K+/Cl− cotransport. In addition, all the above mitogens induced DNA synthesis in the synchronized human fibroblasts. In order to explore the role of the Na+/K+/Cl− cotransport in the mitogenic signal, the effect of two specific inhibitors of the cotransport, furosemide and bumetanide, was tested on cell proliferation induced by the above recombinant growth factors. Bumetanide and furosemide inhibited synchronized cell proliferation as was measured by (a) cell exit from the Go/G1 phase measured by the use of flow cytometry, (b) cell entering the S-phase, determined by DNA synthesis, and (c) cell growth, measured by counting the cells. The inhibition by furosemide and bumetanide was reversible, removal of these compounds, completely released the cells from the block of DNA synthesis. In addition, the two drugs inhibited DNA synthesis only when added within the first 2–6 h of cell release. These results indicate that the effect of these drugs is specific, and is not due to an indirect toxic effect. This study clearly demonstrates that the growth factor-induced activation of the Na+/K+/Cl− cotransport plays a major role in the mitogenic signaling pathway of the human fibroblasts.

Materials and Methods

[1H]Thymidine was purchased from the Nuclear Research Centre, Beer-Shava, Israel. 86Rb+ was purchased from NEN Research Products, Boston, MA. Bumetanide was kindly provided by Laboratoire Leo, B.P. 28500 Vernouillet, Denmark. Phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), 1 propidium iodide, RNAase A, and ouabain were purchased from Sigma, St. Louis, MO. [3H]Thymidine was purchased from the Nuclear Research Centre, Beer-Shava, Israel. 86Rb+ was purchased from NEN Research Products, Boston, MA. Bumetanide was kindly provided by Laboratoire Leo, B.P. 28500 Vernouillet, Denmark. Phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), propidium iodide, RNAase A, and ouabain were purchased from Sigma, St. Louis, MO.

1. Abbreviations used in this paper: bFGF, basic fibroblast growth factor; IGF-1, insulin-like growth factor-1; TGFα, transforming growth factor α; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.
**Table I. Effect of Different Mitogens on the Bumetanide-sensitive Rb+ Influx in Synchronized Cells**

| Addition | Bumetanide-sensitive Rb+ influx (nmol/min/10^6 cells) | Percent |
|----------|-----------------------------------------------------|---------|
| -        | 0.57 ± 0.0                                          | 100     |
| FCS (10%)| 2.39 ± 0.20                                         | 419     |
| PDGF (1 ng/ml)| 1.08 ± 0.09                                         | 190     |
| FGF (1 ng/ml)| 1.27 ± 0.02                                         | 223     |
| TPA (1 ng/ml)| 1.34 ± 0.12                                         | 235     |
| IGF-1 (100 ng/ml)| 1.38 ± 0.38                                         | 242     |
| TGFα (100 ng/ml)| 0.87 ± 0.07                                         | 153     |

Cells were seeded in 15-mm dishes (10,000 cells/dish). After 2 d, the cells were washed and the medium was replaced with DMEM containing 0.2% FCS and 20 mM Hepes. 2 d later the cells were released by the addition of MEM, buffered with Hepes-Tris (40 mM, pH 7.0), the indicated mitogens, and [3H]Rb+ (5 Ci/ml) in the absence and presence of ouabain (0.5 mM) and bumetanide (10 μM) for 6 min at 37°C. Bumetanide-sensitive Rb+ influx was measured and calculated as described in Materials and Methods. Cells were counted at the time of the experiments by coulter counter, and Rb+ fluxes are expressed per 10^6 cells.

Chased from Sigma Chemical Co., St. Louis, MO. Fibroblast growth factor (bFGF), PDGF, insulin-like growth factor-1 (IGF-1), and transforming growth factor α (TGFα), were kindly provided by PeproTech, Inc., Princeton Business Park, Rocky Hill, NJ. α-Thrombin was kindly provided by Dr. R. Bar-Shavit, Department of Oncology, Hadassah University Hospital.

**Cell Cultures**

Primary human skin fibroblasts were kindly provided by Dr. S. Yatsiv, Department of Pediatrics, Hadassah University Hospital. Cells were grown in DMEM, supplemented with 10% fetal calf serum and 4 mM glutamine. This medium was replenished every 2 d. For the arrest of the cell in the G0/G1-phase of the cell cycle, the cells were seeded in 10,000/15-mm dishes. After 2 d the cells were washed once, and the medium was replaced with DMEM, supplemented with 0.2% FCS, 4 mM glutamine and 20 mM Hepes for two more days.

**Rb+ Influx Measurements**

Bumetanide-sensitive Rb+ influx was measured as reported (Panet et al., 1982). In brief, Rb+ influx in the presence of ouabain (0.5 mM) and bumetanide (10 μM) was subtracted from ouabain-resistant Rb+ influx, and taken as bumetanide-sensitive Rb+ influx. Each Rb+ influx measurement was conducted in triplicate cultures and the results (± SEM) were expressed for 10^6 cells.

**DNA Synthesis**

G0/G1; quiescent cells were released by the addition of FCS (10%) or the indicated mitogens, introduced directly into the arrest medium in the presence of [3H]thymidine (0.5 μCi/ml). All thymidine incorporation measurements of this study were performed in the incubator, in the presence of CO2/HCO3-. Trichloroacetic acid-insoluble radioactivity was determined after 48 h after the release.

**Staining the DNA with Propidium Iodide for Flow Cytometry**

Cells trypsinized and washed twice with cold PBS, were suspended in cold PBS (2 × 10^6 cells/ml), and fixed by adding slowly, under gentle stirring, 3 ml cold ethanol (95%). The suspension was kept in a refrigerator (4°C) for further analysis. Before analysis, the cell suspension was centrifuged (1,000 rpm, 5 min), the pellet was suspended in PBS (810 μl) and RNase A (10 mg/ml, 90 μl) was added. After 30 min of incubation at 37°C, propidium iodide (0.5 mg/ml, 90 μl) was added. The samples were kept on ice until the analysis was conducted. Samples were run in the flow cytometer between 15 min and 3 h after the addition of propidium iodide. The flow cytometry used was FACSTAR Plus (Becton Dickinson & Co., Oxnard, CA). The percentages of cells in the different phases of the mitotic cycle were calculated using the Cellfit R software (Becton Dickinson & Co.).

**Results**

**The Bumetanide-sensitive Rb+ Influx Is Stimulated by Different Mitogens in Synchronized Human Fibroblasts**

Addition of FCS to quiescent human fibroblasts resulted in a dramatic fourfold stimulation of the bumetanide-sensitive Rb+ influx (Table I). The following mitogens: PDGF, bFGF, TPA (all at 1 ng/ml), TGFα, and IGF-1 (both at 100 ng/ml), all stimulated the bumetanide-sensitive Rb+ influx by about twofold of the control.

**Effect of Bumetanide on DNA Synthesis Induced by α-Thrombin in the Synchronized Human Fibroblasts**

We next examined whether stimulation of the Na+/K+/Cl-cotransport is essential for the mitogenic signal. To this end, we measured the effect of bumetanide, a specific inhibitor of the Na+/K+/Cl-cotransport, on synchronized cell entry into S-phase induced by α-thrombin. Human fibroblasts were arrested by serum deprivation, and α-thrombin was added in the presence of different bumetanide concentrations. The incorporation of [3H]thymidine into trichloroacetic acid-insoluble material was determined after 48 h release in the presence of CO2/HCO3-. As seen in Fig. 1, bumetanide inhibited thymidine incorporation induced by α-thrombin. The effect of bumetanide was dose dependent; at 100 μM concentration it inhibited a noteworthy fraction of thymidine incorporation into DNA (50%) induced by α-thrombin (Fig. 1). However, the basal level of thymidine incorporation was not affected (Fig. 1). Based on this result, bumetanide was added at 100 μM in all the experiments described in this study.

**Effect of Bumetanide and Furosemide on Cell Growth and Exit from the G0/G1-Phase of the Cell Cycle**

To analyze whether bumetanide does not just inhibit the synthesis of DNA, but in fact blocks cell proliferation, we measured cell replication by direct counting. Quiescent cells, which were starved for 48 h, were released by the addition of PDGF (10 ng/ml) or bFGF (1 ng/ml) in the absence or presence of bumetanide. After 3 d the cells (grown in the presence of CO2/HCO3-) were trypsinized and counted in a
Figure 2. Effect of bumetanide on cell proliferation induced by PDGF and FGF. Quiescent human skin fibroblasts were released by the addition of PDGF (10 ng/ml) or FGF (1 ng/ml) in the absence or presence of bumetanide (100 μM). After 3 d the cells were counted by Coulter counter.

Coulter counter. As seen in Fig. 2, bumetanide (100 μM) added to synchronized human fibroblasts, inhibited cell growth induced by either PDGF or bFGF. Similar results were obtained with furosemide (data not shown).

To directly determine the phase in which furosemide or bumetanide blocks cell cycle, the methodology of cytofluorometry was applied. As seen in Fig. 3 A, the synchronized control cells exit the G1 and enter the S-phase at 16 h after serum addition. However, when serum was added in the presence of furosemide, cell exit from the G1-phase was much reduced (Fig. 3 B). At 24 h after serum addition, 60% of the control cells exit the G1-phase, however, when cells are released in the presence of furosemide, only 27% of the cells exit the G1-phase and enter the S-phase (Fig. 3 B). Similar results were obtained with bumetanide (data not shown).

Since three independent techniques suggested that bumetanide and furosemide block cell cycle, we propose that the effect is mediated through inhibition of the Na+/K+/Cl- cotransport.

PDGF and bFGF were found to be potent mitogens of human skin fibroblasts (Fig. 4). Both growth factors, PDGF (10 ng/ml) and bFGF (1 ng/ml) stimulated thymidine incorporation into DNA to a degree similar to that elicited by FCS (Fig. 6). Bumetanide or furosemide inhibited a considerable fraction of the thymidine incorporation induced by either PDGF (40–54%), or bFGF (46–78%). It appears that at high concentrations of bFGF the inhibition of DNA synthesis by bumetanide declined. This phenomenon could also be observed with PDGF but to a lesser extent (Fig. 4). These two inhibitors may either be specific for inhibition of the stimulation of the Na+/K+/Cl- cotransport at the early G0/G1-phase, or they may exhibit an effect on cellular requirements for the Na+/K+/Cl- cotransport during the cell cycle. To resolve this question, we measured the effect of bumetanide when added to the synchronized cultures at different times after the release by PDGF. As seen in Table II addition of bumetanide within the first 2 h of cell release, inhibited thymidine incor-
Table II. Effect of Bumetanide Added at Different Times after PDGF Stimulation on Thymidine Incorporation into DNA

| Addition | Bumetanide addition time h | cpm     | Percent |
|----------|----------------------------|---------|---------|
| PDGF     | 0                          | 9,125 ± 725 | 56      |
| PDGF     | 2                          | 10,140 ± 285 | 62      |
| PDGF     | 6                          | 11,065 ± 865 | 69      |
| PDGF     | 8                          | 12,845 ± 550 | 80      |
| PDGF     | 12                         | 14,130 ± 175 | 88      |

Quiescent human fibroblasts were released by the addition of PDGF (10 ng/ml). Bumetanide (100 µM) was added at the indicated time after PDGF addition. After 48 h trichloroacetic acid-insoluble radioactivity was determined and expressed as cpm/15-mm plate.

poration into DNA by 45%, however, when bumetanide was added 6-12 h after the addition of PDGF, inhibition of thymidine incorporation into DNA declined to 12% (Table II). This result supports the proposal that bumetanide specifically inhibits early events during cell exit from the G₀/G₁ phase, rather than the cells' requirement for the Na⁺/K⁺/Cl⁻ cotransport during the cell cycle.

Effect of Furosemide on the Protein Kinase C-dependent Mitogenic Pathway

As seen in Table I, bumetanide-sensitive Rb+ influx is stimulated by the phorbol ester TPA. This result indicates the involvement of protein kinase C in the stimulation of the Na⁺/K⁺/Cl⁻ cotransport in the human fibroblasts. To analyze whether stimulation of the Na⁺/K⁺/Cl⁻ cotransport through the protein kinase C pathway (Nishizuka, 1984) plays a role in the mitogenic signal, we measured the consequences of inhibiting this transporter on DNA synthesis induced by TPA. The phorbol ester TPA is a relatively weak mitogen for human fibroblasts compared with FCS, PDGF, or bFGF. Nevertheless, TPA at 0.1-10 ng/ml stimulated thymidine incorporation by 4.7-fold (Fig. 5 A). Furosemide inhibited 40-50% of thymidine incorporation induced by TPA, and similar results were achieved by bumetanide (data not shown). This observation suggests that stimulation of the Na⁺/K⁺/Cl⁻ cotransport by protein kinase C-dependent pathway is involved in this mitogenic pathway.

Effect of Furosemide on Protein Kinase C-independent Mitogenic Signal

EGF is mitogenic to human and mouse fibroblasts through a protein kinase C-independent pathway. In a recent paper we have demonstrated that the Na⁺/K⁺/Cl⁻ cotransport is stimulated by both a protein kinase C-dependent mechanism and by the protein kinase C-independent pathway of the mitogenic signal (Panet and Atlam, 1990). To analyze whether stimulation of the Na⁺/K⁺/Cl⁻ cotransport through the protein kinase C-independent pathways is also involved in the mitogenic signal, we measured the effect of furosemide on DNA synthesis induced by TGFα (Blackshear et al., 1985; Coughlin et al., 1985; Besterman et al., 1986). As seen in Fig. 6, TGFα is mitogenic to these cells, and furosemide at 1 mM inhibited a large portion (35-55%) of the mitogenic response to TGFα. This result suggests that stimulation of the Na⁺/K⁺/Cl⁻ cotransport by the protein kinase C-independent pathway is also an essential part for this mitogenic pathway.

Bumetanide Inhibited DNA Synthesis Induced by FCS in the Human Fibroblasts

When the synchronized cells were released by FCS (10%), thymidine incorporation into DNA was inhibited by bumetanide to a smaller extent (in four independent experiments, 29 ± 11% inhibition was obtained by bumetanide) than when the cells were released by the defined mitogens (Figs. 4 and 5). To investigate the reason for the weak effect of bumetanide on thymidine incorporation induced by 10% FCS, we repeated the experiment but with lower concentrations of FCS. Quiescent human fibroblasts were stimulated by increasing concentrations of FCS, in the absence and presence of bumetanide. 48 h later, the incorporation of [³H]thymidine into DNA was determined. As seen in Fig. 6, the extent of
The question of whether the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport plays a role in the mitogenic signal pathway is the subject of this study. We have shown here that the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport is dramatically stimulated by either fetal calf serum, or by purified recombinant growth factors, added to quiescent G0/G1 human skin fibroblasts. Furthermore, we have shown that two specific inhibitors of the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport, furosemide and bumetanide, inhibited 50–80\% of DNA synthesis induced by the various mitogens. Similarly, the two drugs inhibited cell growth and cell exit from the G0/G1 phase of the cell cycle, induced by the growth factors. The following mitogens were found to stimulate the bumetanide-sensitive Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport in quiescent human fibroblasts: bFGF, PDGF, α-thrombin, IGF-I, TGF-α, and the phorbol ester TPA. To our knowledge, this is the first evidence that specific inhibitors of the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport block cell proliferation. This study, therefore, indicates that the growth factor-induced activation of the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport plays a major role in the mitogenic signal pathway. The finding that bumetanide inhibited DNA synthesis induced by both mitogens which stimulate protein kinase C (bFGF, PDGF, α-thrombin; Blackshear et al., 1985; Coughlin et al., 1985; Besterman et al., 1986; L’Allemand et al., 1986) and those which do not activate protein kinase C (TGFα and IGF-1; Blackshear et al., 1985; Coughlin et al., 1985), implies that stimulation of the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport has an essential role in the two mitogenic pathways. The degree of cell-cycle inhibition by bumetanide and furosemide depends on the concentration of the specific mitogen. This is most distinct with bFGF and FCS (see Figs. 4 and 6). It seems quite clear that increasing the mitogen concentrations, overcomes the inhibition by bumetanide or furosemide. This phenomenon is especially dominated in cells induced by FCS. This might indicate that high concentrations of the mitogen induce other transport systems which replace the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport.

The following evidence indicates that the two drugs block cell exit from the G0/G1-phase of the cell cycle by inhibiting the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport. (a) Bumetanide and furosemide inhibited thymidine incorporation into DNA only when added within the first 2–6 h of cell release. (b) In correlation to its effect on thymidine incorporation, the two drugs inhibited also cell growth (Fig. 2) and cell exit from the G0/G1-phase, as measured by flow cytometry (Fig. 3). (c) The two drugs reversibly inhibited thymidine incorporation into DNA induced by different mitogens (Figs. 1 and 4–7).

The finding that bumetanide and furosemide inhibited similarly cell proliferation, cell exit from the G0/G1-phase and thymidine incorporation, excludes the possibility that the two drugs repress thymidine uptake, rather than thymidine incorporation into DNA. In addition, it also excludes the contingency that it slows down DNA synthesis rather than blocking it. Inhibition of cell proliferation by the two drugs might exhibit cell requirement for the Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport during the cell cycle, rather than specific inhibition of the cotransport stimulation by the mitogens. The finding that bumetanide inhibited thymidine incorporation within the first hours of PDGF addition, whereas 8–12 h after the mitogenic stimulation, the inhibition declined, implies that this inhibitor is specific for a direct mitogen response. The finding that bumetanide only slightly inhibited (12% inhibition) thymidine incorporation when added 12 h after PDGF addition, together with the finding that the inhibition is reversible, excludes the possibility that it has a toxic rather than specific effect.

The bumetanide-sensitive Na\(^+\)/K\(^+\)/Cl\(^-\) cotransport was reported to be dramatically stimulated by serum growth factors in immortal cell lines such as 3T3 mouse cells, or in hamster fibroblasts (Tupper et al., 1977; Panet et al., 1982, 1983; Panet, 1985; Amsler et al., 1985; Paris and Pouys
segur, 1986; Panet et al., 1986a,b, 1989). On the other hand, one report has suggested that this transporter is only slightly stimulated in human diploid fibroblasts (Owen and Prastein, 1985). Recently we have established optimal conditions for arresting human skin fibroblasts at the G0/G1-phase of the cell cycle (Panet and Atlan, 1990). Under these conditions, the Na+/K+/Cl- cotransport is two- to threefold stimulated by either fetal calf serum or purified recombinant growth factors. The observation that this transporter in human fibroblasts was only slightly stimulated by growth factors (Owen and Prastein, 1985) could be due to the insufficient period of serum starvation (4 h).

In summary, we have shown that bumetanide or furosemide, added to quiescent human skin fibroblasts, inhibited 50-80% of cell proliferation induced by different mitogens. This study demonstrates that the growth factor stimulation of the Na+/K+/Cl- cotransport plays an essential role in the mitogenic signal of the human fibroblasts.

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