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 G_0/G_1 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 G_0/G_1 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.

One of the earliest responses of quiescent cells to a mitogenic signal is activation of Na^+ influx (Rozengurt, 1986; Rozengurt and Mendoza, 1986). The bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport was shown to be dramatically stimulated by the addition of serum growth factors to quiescent cells (Tupper et al., 1977; Panet et al., 1982, 1983; Panet, 1985; Amsler et al., 1985; Paris and Pouyssegur, 1986; Panet et al., 1986*a*, 1989). The question of whether the $Na^+/K^+/Cl^-$ cotransport has an essential role in the mitogenic response is still unsolved. Several groups have observed that inhibition of the $Na^+/K^+/Cl^-$ cotransport affected only slightly the initiation of DNA synthesis (Owen and Prastein, 1985; Amsler et al., 1985; Paris and Pouyssegur, 1986). It has therefore been proposed that the $Na^+/K^+/Cl^-$ cotransport does not play a major role in the mitogenic signal (Amsler et al., 1985; Paris and Pouyssegur, 1986). Most of these studies, however, were carried out with immortal rodent cell lines. Nevertheless, the response of normal diploid human fibroblasts, rather than immortal cell lines to growth factors, may be different and more relevant to the normal control of cell proliferation.

Recently, we have investigated the optimal conditions for the arrest of human skin fibroblasts. Optimal cell density, duration of serum deprivation, and the serum concentration in the arrest medium, were analyzed. We have shown that in cultures of human skin fibroblasts arrested under optimal conditions, the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport is stimulated by two- to threefold after the addition of fetal calf serum, or purified recombinant growth factors (Panet and Atlan, 1990). In the present study, we analyzed, with the use of specific inhibitors furosemide and bumetanide, whether stimulation of the $Na^+/K^+/Cl^-$ cotransporter by serum growth factors is essential for human fibroblast proliferation. Our results indicate that the growth factors-induced stimulation of the $Na^+/K^+/Cl^-$ cotransport is an essential part of the mitogenic signal of the human fibroblasts.

Materials and Methods

[^{3}H]Thymidine was purchased from the Nuclear Research Centre, Beer-Sheva, Israel. $^{86}Rb+$ 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 pur-

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 ^{86}Rb+ (5 μCi/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. Yatziv, 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 G_0/G_1-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

G_0/G_1 quiescent cells were released by the addition of FCS (10%) or the indicated mitogens, introduced directly into the arrest medium in the presence of [^{3}H]thymidine (0.5 μCi/ml). All thymidine incorporation measurements of this study were performed in the incubator, in the presence of CO_2/HCO_3. Trichloroacetic acid-insoluble radioactivity was determined 48 h after the release.

Staining the DNA with Propidium Iodide for Flow Cytofluorometry

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.).

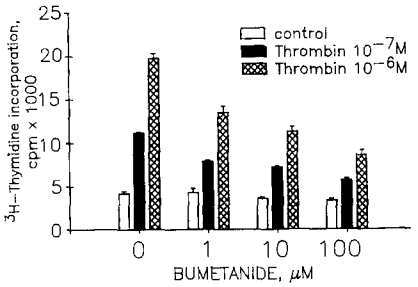

Figure 1. Effect of bumetanide on thymidine incorporation into DNA induced by α-thrombin. Human skin fibroblasts were seeded and serum deprived for 2 d, as described in Materials and Methods. Bumetanide at increasing concentrations was added directly into the arrest medium in the presence and absence of α-thrombin with [^{3}H]thymidine (0.5 μCi/ml). After 48 h trichloroacetic acid-insoluble radioactivity was determined and expressed as cpm/15-mm plate.

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 [^{3}H]thymidine into trichloroacetic acid-insoluble material was determined after 48 h release in the presence of CO_2/HCO_3. 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 G_0/G_1-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 CO_2/HCO_3) were trypsinized and counted in a

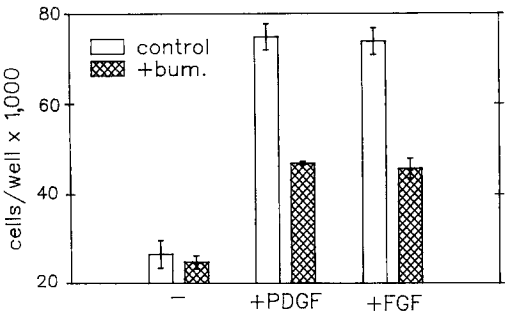

Figure 2. Effect of bumetanide on cell proliferation induced by PDGF and FGF. Quiescent human skin fibroblasts were released by the addition of PDGF (10 ng/ml) or FGF (1 ng/ml) in the absence or presence of bumetanide (100 μM). After 3 d the cells were counted by Coulter counter.

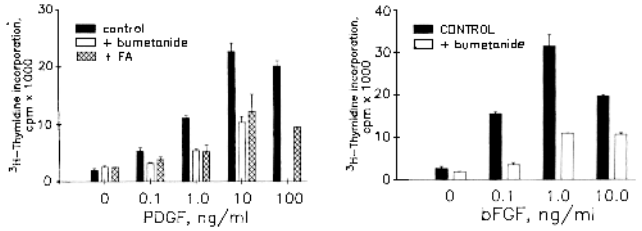

Figure 4. Effect of furosemide and bumetanide on thymidine incorporation induced by PDGF and bFGF. Human skin fibroblasts were seeded and serum deprived for 2 d, as described in Materials and Methods. The indicated concentrations of PDGF or bFGF were added directly into the arrest medium with [^{3}H]thymidine (0.5 μCi/ml), in the absence or presence of furosemide (*FA*, 1 mM) or bumetanide (100 μM). After 48 h, trichloroacetic acid-insoluble radioactivity was determined and expressed as cpm/15-mm plate. [^{3}H]Thymidine incorporation into DNA by the addition of FCS (10%) was 30,000 ± 2,000 cpm/15-mm well.

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 G_1 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 G_1-phase was much reduced (Fig. 3 *B*). At 24 h after serum addition, 60% of the control cells exit the G_1-phase, however, when cells are released in the presence of furosemide, only 27% of the cells exit the G_1-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 G_0/G_1-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-

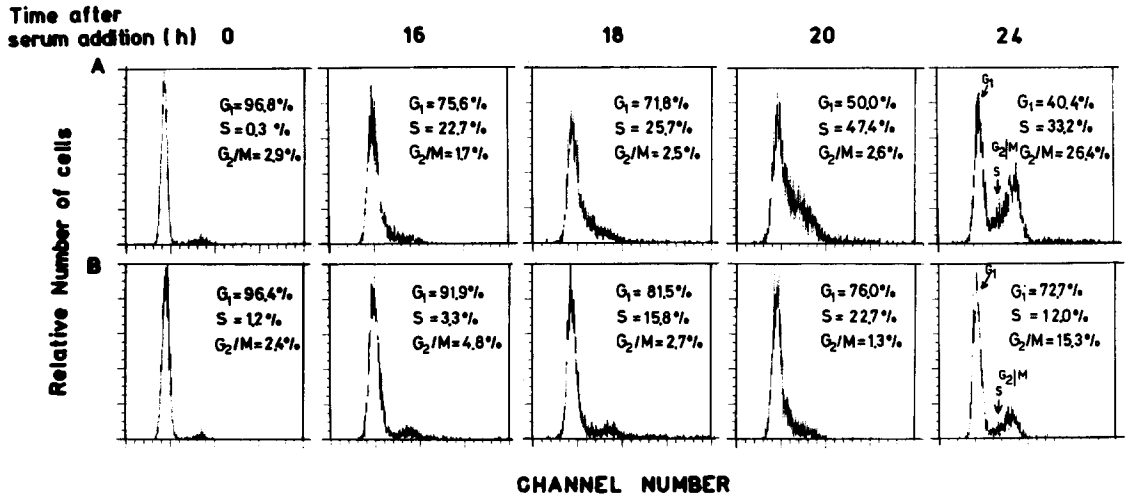

Figure 3. Effect of furosemide on the cell cycle of synchronized cells induced by FCS. Human skin fibroblasts were seeded and serum deprived for 2 d, as described in Materials and Methods. The quiescent cells were released by the addition of FCS (5%) in the absence and presence of furosemide (1 mM), which was introduced 15 min before serum was added. At the indicated time cells were harvested, washed, fixed, and labeled with propidume iodide as described in Materials and Methods. Fluorescence distribution of the cells was measured using flow cytometer as described in Materials and Methods. In all samples 5,000 nuclei were analyzed by flow cytometry.

Table II. Effect of Bumetanide Added at Different Times after PDGF Stimulation on Thymidine Incorporation into DNA

Addition	Bumetanide addition time	Thymidine incorporation	
		cpm	Percent
	h		
–	–	3,390 ± 870	
PDGF	–	16,185 ± 325	100
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.

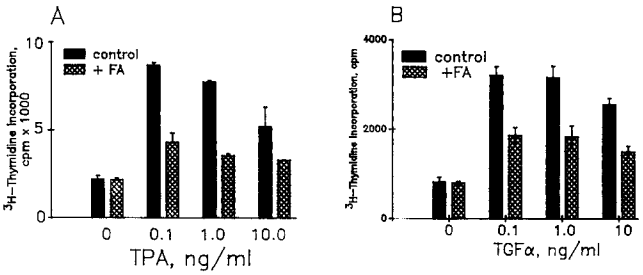

Figure 5. Effect of furosemide on thymidine incorporation induced by TPA and TGFα. Human skin fibroblasts were seeded and serum deprived for 2 d, as described in Materials and Methods. The indicated concentration of TPA (*A*) or TGFα (*B*) was added directly into the arrest medium with [^{3}H]thymidine (0.5 μCi/ml), in the absence or presence of furosemide (*FA*, 1 mM). 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 G_0/G_1 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 Atlan, 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. 5 *B*, 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 [^{3}H]thymidine into DNA was determined. As seen in Fig. 6, the extent of

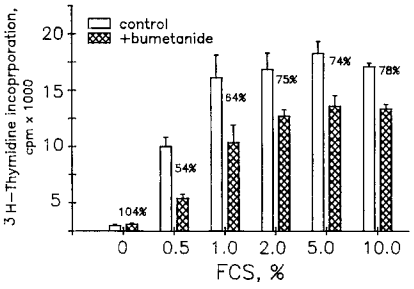

Figure 6. Effect of bumetanide and furosemide on thymidine incorporation induced by FCS. Human skin fibroblasts were seeded and serum deprived for 2 d, as described in Materials and Methods. The indicated concentrations of FCS were added directly into the arrest medium with [^{3}H]thymidine (0.5 μCi/ml) in the absence or presence of bumetanide (100 μM). After 48 h trichloroacetic acid-insoluble radioactivity was determined and expressed as cpm/15-mm plate. Thymidine incorporation into DNA at any designated FCS concentration, was taken as 100%, and the percentage of DNA synthesis in the presence of bumetanide was calculated and marked above the suitable bars.

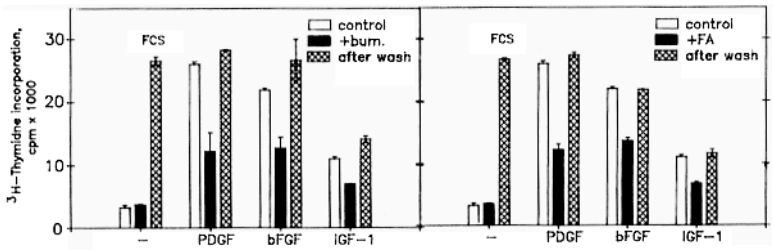

Figure 7. Reversible inhibition of DNA synthesis by furosemide and bumetanide in synchronized human skin fibroblasts. G_0/G_1-phase-arrested human skin fibroblasts were released by the addition of PDGF (10 ng/ml), bFGF (1 ng/ml), or IGF-1 (100 ng/ml) in the absence (*open bars*) or presence (*solid bars*) of furosemide (*FA*, 1 mM) or bumetanide (*bum.*, 100 μM) with [^{3}H]thymidine (0.5 μCi/ml). After 48 h trichloroacetic acid-insoluble radioactivity was determined and expressed as cpm/15-mm plate. In parallel, other cultures were similarly released in the presence of the drugs for 48 h, then washed and fed with fresh medium containing the same mitogens without the drugs in the presence of [^{3}H]thymidine (0.5 μCi/ml). After a further 48-h trichloroacetic acid-insoluble radioactivity was determined and expressed as cpm/15-mm plate (*crosshatched bars*). Cultures that were exposed to furosemide or bumetanide in the absence of any drug in the first part of the experiment were released in the second step by the addition of FCS (10%). Note that PDGF and FGF stimulated thymidine incorporation into DNA to a degree similar to that elicited by FCS.

bumetanide inhibition of thymidine incorporation is dependent on FCS concentrations. It appears that at high concentrations of FCS, the inhibition of DNA synthesis by bumetanide declined. This phenomenon might indicate that high concentrations of FCS stimulate other transmembrane pathways which are not affected by bumetanide.

Reversible Inhibition of Cell Proliferation by Furosemide and Bumetanide

To test whether inhibition of DNA synthesis by these compounds is due to a toxic effect, cell cultures which had been preincubated for 48 h with PDGF, bFGF, or IGF-1, in the presence of furosemide or bumetanide, were washed and fed with a fresh medium containing the same mitogens, but without the inhibitors. 48 h later we measured cell proliferation by thymidine incorporation into DNA. The inhibition by furosemide and bumetanide was reversible; removal of these compounds completely released the cells from the block of DNA synthesis (Fig. 7).

Discussion

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 demonstrated here, that the $Na^+/K^+/Cl^-$ cotransport is dramatically stimulated by either fetal calf serum, or by purified recombinant growth factors, added to quiescent G_0/G_1 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 G_0/G_1 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-1, 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'Allemain 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 G_0/G_1-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 G_0/G_1-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 G_0/G_1-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., 1986*a*,*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 G_0/G_1-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.

We wish to thank Dr. Amos Panet for his helpful comments and critical reading of the manuscript, Dr. S. Yatziv for supplying the human skin fibroblasts, Dr. R. Bar-Shavit for supplying the α-thrombin, and Dr. E. Fibach for helping us using the FACS flow cytometer and evaluating the results of the cell cycle study. We thank Miriam Markus for her excellent technical assistance.

This work was supported by the Israel Cancer Research Fund and by the Israel Cancer Association.

Received for publication 14 February 1991 and in revised form 3 April 1991.

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