K$^+$ efflux in NIH mouse 3T3 cells and transformed derivatives: Dependence on extracellular Ca^{2+} and phorbol esters

(protein kinase C/ion channels/simian virus 40/polyoma virus)

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ABSTRACT   In culture medium deficient in Ca^{2+}, NIH mouse 3T3 cells lose K$^+$, gain Na$^+$, and stop growing. A marked increase in the rate of K$^+$ efflux accounts for this loss; Na$^+$,K$^+$-ATPase pump activity increases but does not fully compensate for enhanced K$^+$ efflux. Phorbol esters and cycloheximide inhibit K$^+$ loss in Ca^{2+}-deficient medium. Phorbol esters inhibit K$^+$ efflux from human fibroblasts as well, even at physiological levels of Ca^{2+}. Two cell lines derived from NIH-3T3, one transformed by a simian virus 40 deletion mutant, the other by the polyoma virus oncogene encoding the middle-sized tumor antigen, retain K$^+$ and can multiply in medium with low Ca^{2+}. Efflux of K$^+$ from these cells is relatively insensitive to reduced Ca^{2+} concentration, phorbol esters, and cycloheximide. The results suggest the following hypothesis: a channel, nonselective for K$^+$ and Na$^+$, opens when NIH-3T3 cells are in Ca^{2+}-deficient medium; the channel is controlled by the receptor for phorbol ester (protein kinase C) and may also be regulated by a short-lived protein.

Ordinarily, untransformed mammalian cells will not grow if the concentration of Ca^{2+} in the culture medium is decreased from normal levels, above 1 mM, to less than 0.05 mM (1). Among the reported effects of Ca^{2+}-deficient media that may account for growth inhibition, loss of cell K$^+$ has been noted. This loss has been attributed to a passive "leak" of K$^+$ (2–7), implying that the cytoplasmic membrane becomes permeable.

Diverse types of transformed cells, however, can grow in Ca^{2+} concentrations as low as 0.01–0.05 mM. Although this peculiar feature of transformation was described some time ago (1, 8), no definitive explanation has emerged of why transformed but not untransformed cells are Ca^{2+}-independent. The possibility of a connection between the capacity to grow in low Ca^{2+} and transformation has been strengthened by a report (9) of a new class of transformants obtained after infection of Swiss mouse 3T3 cells with simian virus 40 (SV40). This class, selected for the ability to grow in reduced Ca^{2+}, also showed typical characteristics of transformation. In the same study, NIH mouse 3T3 cells transformed with SV40 were found to grow better in reduced Ca^{2+} than untransformed NIH-3T3 cells (9). That report, and previous findings that Ca^{2+}-deficient media cause loss of cell K$^+$, prompted the present study of K$^+$ transport in NIH-3T3 cells and two transformed derivatives.

MATERIALS AND METHODS

Cells, Chemicals, and Media. NIH-3T3 cells were obtained from R. A. Weinberg (Massachusetts Institute of Technology, Cambridge, MA) and subcloned. SV-NIH-3T3 cells, obtained by transfection of NIH-3T3 with dl1883 (10), a SV40

deletion mutant that lacks the sequence encoding the small tumor (t) antigen, were from D. M. Livingston (Harvard Medical School, Cambridge, MA). mT-NIH-3T3 cells (clone 336a), from L. Raptis and T. L. Benjamin, were derived by transfection of NIH-3T3 with a plasmid containing the polyoma virus middle-sized tumor (mT)-antigen gene controlled by the dexamethasone-inducible promoter of mouse mammary tumor virus (11) (addition of dexamethasone was not necessary to elicit the transport properties described below, presumably because background levels of mT gene expression were sufficient). Swiss 3T3a, a subclone of CCL92 (American Type Culture Collection), was isolated in this laboratory. Human foreskin fibroblasts (15th to 25th passage) were from Mary Hitchcock Memorial Hospital (Hanover, NH), and MRC-5 human lung fibroblasts were from Flow Laboratories. ^{86}Rb$^+$ and [^{14}C]urea were from New England Nuclear; bumetanide was from Hoffmann–La Roche; and 4β-phorbol 12-myristate 13-acetate (PMA), 4α- and 4β-phorbol 12,13-didecanoate, ouabain, cycloheximide, puromycin, Hepes, nifedipine, and tetraethylammonium chloride were from Sigma. Cultures were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C in a 5% CO$_2$/95% air mixture and were used at cell densities between 4 × 10^5 and, just as cultures reached confluence, 1.8 × 10^6 per 30-cm^2 plastic dish. Ca^{2+}-deficient medium (DMEM-a) had the components of DMEM except that vitamins (containing calcium pantothenate) were at one-fourth strength and Ca^{2+} was omitted. Dialyzed serum was prepared by dialysis of fetal bovine serum against 10 volumes of 0.9% NaCl with four daily changes. Ca^{2+} electrode measurements (provided by P. A. Friedman) of DMEM-a with 10% dialyzed serum showed a Ca^{2+} content of less than 2 μM. Salts/Hepes medium contained 5 mM KCl, 0.8 mM MgCl$_2$, 154 mM NaCl, 5.6 mM glucose, 0.04 mM phenol red, and 20 mM Hepes (pH 7.2).

Ion Concentrations and Fluxes. Cell numbers and relative volumes were determined on detached cells in a Coulter model ZBI with Channelyzer (Coulter). Intracellular water space of attached cells was determined by [^{14}C]urea distribution, with reproducibility between dishes of about 10%, as described (12). Intracellular K$^+$ and Na$^+$ concentrations were calculated from cell water space and from measurements of ion content by flame photometry (Instrumentation Laboratories, Lexington, MA). Flux measurements were initiated by rapidly rinsing cultures four times with new medium, warmed in advance to 37°C in a CO$_2$/air atmosphere (for DMEM-a) or in air (for salts/Hepes medium), with a final addition of 2.5 or 3 ml of medium. When PMA (from 30 μM stocks in dimethyl sulfoxide) or cycloheximide were in DMEM-a or salts/Hepes medium, they were also added in advance of medium change (see figure legends). Bumetanide was added from 50 mM stocks in dimethyl sulfoxide 10 min before change of medium. Dimethyl sulfoxide alone (0.2%)

Abbreviations: PMA, 4β-phorbol 12-myristate 13-acetate; SV40, simian virus 40.

had no effect on flux rates; concentrations chosen for PMA, cycloheximide, and bumetanide produced maximal effects.

Efflux of K^+ was measured in two ways. In the first, cell K^+ content was measured by flame photometry on sample dishes by rinsing in iced 0.1 M $MgCl_2$ as described (12); ouabain (2 mM for mouse cells, 50 μM for human cells) was present to block the Na^+,K^+-ATPase pump. Cell K^+ and Na^+ were measured in each sample, but since the sum of the two ions remained nearly constant (within 10%) under all experimental conditions, data for K^+ alone are given, except in Fig. 1. In the second method, $^{86}Rb^+$ (2–3 μCi/ml; 1 Ci = 37 GBq), a tracer for K^+, was added to cultures for 3 hr and, after change of medium, 100 to 200 μl samples were taken at intervals for liquid scintillation counting. After the last sample, cultures were rinsed in iced 0.1 M $MgCl_2$ and the extract was used for $^{86}Rb^+$, K^+, and Na^+ determinations. For influx measurements, medium was changed to serum-supplemented DMEM-a with Ca^{2+} and with 2 mM ouabain in alternate dishes. After 6 min, $^{86}Rb^+$ was added; 6 min later, cultures were rinsed with $MgCl_2$ and the $^{86}Rb^+$ content of cells was measured. The ouabain-sensitive fraction of $^{86}Rb^+$ influx was taken as a measure of active K^+ transport.

Each experiment was repeated at least twice; each data point given is an average of duplicate determinations. For flame photometric measurements, the standard deviation of duplicate samples, as percent of the mean value, was 3% for K^+ and 4% for Na^+. For $^{86}Rb^+$ samples, the standard deviation was 3% of the mean.

RESULTS

NIH-3T3 Cells in Ca^{2+}-Deficient Medium Lose K^+ and Gain Na^+. When NIH-3T3 cells were in medium with 0.05 mM Ca^{2+}, more than half of cell K^+ was lost in 30 min (Fig. 1). The levels of K^+ and Na^+ changed in opposite directions, with the sum of the two ions remaining nearly constant. At 0.2 mM Ca^{2+}, cells maintained high levels of K^+. After 20 min, cells in medium with low (0.025–0.05 mM) or high (1.2 mM) Ca^{2+} showed no detectable differences in volume (data not shown). Hence the intracellular K^+ and Na^+ concentrations were presumably proportional to the relative changes shown in Fig. 1. Cells in 0.05 mM Ca^{2+} did not grow; those in 0.2 mM Ca^{2+} proliferated almost as rapidly as those in 1.2 mM Ca^{2+} (data not shown).

NIH-3T3 Cells in Ca^{2+}-Deficient Medium Show Increased K^+ Efflux. Net loss of cell K^+ in Ca^{2+}-deficient medium (Fig. 1) could be due to either decreased influx or increased efflux, and hence these were assessed separately (Table 1).
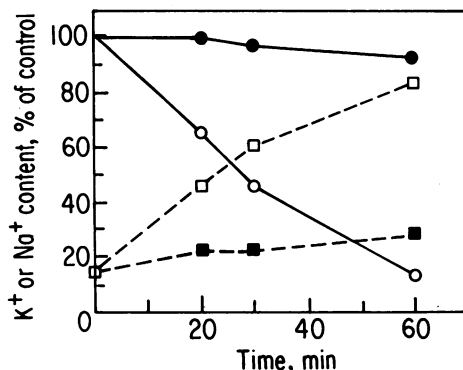

FIG. 1. K^+ and Na^+ content of NIH-3T3 cells in Ca^{2+}-deficient medium. At time zero, medium was changed to DMEM-a with 5% dialyzed serum and 0.05 or 0.2 mM Ca^{2+}. At intervals, cultures were processed for analysis of K^+ and Na^+ content. Values are given as percent of K^+ in control cultures whose medium was not changed. For 0.05 mM Ca^{2+}: ○, K^+; □, Na^+. For 0.2 mM Ca^{2+}: ●, K^+; ■, Na^+.

Table 1. K^+ efflux and influx in NIH-3T3 cells in high- and low-Ca^{2+} media

Measurement	K^+, nmol/min	
	1.2 mM Ca^{2+}	0.05 mM Ca^{2+}
Efflux	6.5	20.4
Influx	5.2	8.7
Efflux − influx	1.3	11.7

Medium was changed to DMEM-a with 5% dialyzed serum and Ca^{2+} as shown. For K^+ efflux, ouabain was present; for influx, medium contained ouabain in alternate dishes (see *Materials and Methods*). Cell K^+ content of control cultures was 470 nmol.

Influx was calculated from the ouabain-sensitive fraction of $^{86}Rb^+$ taken up by cells in 6 min at 1.2 or 0.025 mM Ca^{2+}. Efflux was measured over 20 min to provide sufficient time for changes in K^+ content to be measured with accuracy.

In control cultures with Ca^{2+} at 1.2 mM, efflux and influx, as expected, matched closely. The small difference found (1.3 μmol/min) is within experimental error but might also be explained by differences in the methods of measuring efflux and influx. In 0.05 mM Ca^{2+}, however, the difference between efflux and influx was large. Efflux increased about 3-fold, but influx increased only about 50% over control rates. Hence, net loss of K^+ (and gain of Na^+) in low-Ca^{2+} medium is due to a large increase in efflux that is not sufficiently balanced by a modest increase in influx. A substantial increase in efflux rate in low-Ca^{2+} medium was consistently found: in seven experiments the average decrease in K^+ content in 20 min was 30.8 ± 4.1% (mean ± SEM) when Ca^{2+} was 1.2 mM, but K^+ content decreased 85.7 ± 4.8% when Ca^{2+} was 0.025–0.05 mM. Since intracellular K^+ was 147 ± 11 mM (mean ± SEM, $n = 4$) in control cultures, these percent decreases resulted in K^+ concentrations, after 20 min, of about 100 mM, for medium with 1.2 mM Ca^{2+} and 20 mM for media with 0.025–0.05 M Ca^{2+}.

$^{86}Rb^+$ Efflux from NIH-3T3 Cells in Ca^{2+}-Deficient Medium Has Three Components. Increased K^+ efflux appears to account for loss of cell K^+ in Ca^{2+}-deficient medium. To assess the rapidity with which K^+ efflux increases in Ca^{2+}-deficient medium, efflux of $^{86}Rb^+$ from prelabeled cells was measured. For cells in 0.025 mM Ca^{2+}, the percentage of $^{86}Rb^+$ released by the earliest time of sampling was nearly twice that in 1.2 mM Ca^{2+} (Fig. 2). Bumetanide, which blocks the KCl/NaCl cotransport system, inhibited part of the $^{86}Rb^+$ efflux, and PMA (30 nM) produced even further inhibition. At 1.2 mM Ca^{2+}, bumetanide also reduced efflux, but PMA caused no further reduction. The effect of PMA was the same when tested from 10–100 nM (data not shown).

$^{86}Rb^+$ efflux appears to have three components: one inhibited by bumetanide at both low and high Ca^{2+} concentrations, as reported previously for another cotransport inhibitor, furosemide (3); a second inhibited by PMA at low but not at high Ca^{2+}; and a third not inhibited by either PMA or bumetanide. Since the bumetanide-inhibitable cotransport system can carry out both net movement of KCl/NaCl and exchange of ions without net transport (13), the effect of bumetanide on net K^+ efflux could not be assessed from $^{86}Rb^+$-efflux measurements alone. Hence further experiments were carried out to determine, by flame photometry, only net efflux of K^+.

PMA Inhibits K^+ Efflux from NIH-3T3 Cells in Ca^{2+}-Deficient Medium. NIH-3T3 cells were incubated in low- or high-Ca^{2+} medium with ouabain in the absence or presence of 30 nM PMA. In low-Ca^{2+} medium, K^+ content after 20 min was 40% of control values (Fig. 3). The rate of K^+ loss at low Ca^{2+} in this experiment was less than in the experiment of Table 1 but was nevertheless clearly higher than at
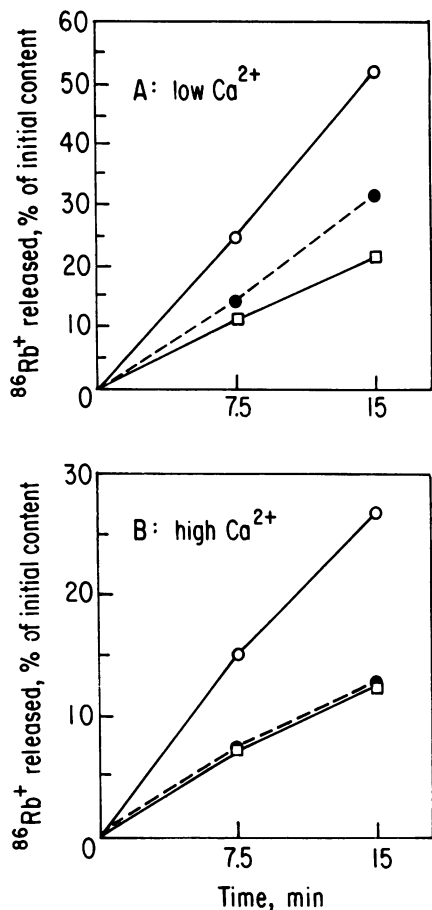
FIG. 2. $^{86}Rb^+$ efflux from NIH-3T3 cells in media with high and low Ca^{2+}. $^{86}Rb^+$ was added to cultures; after 3 hr medium was changed to DMEM-a with 5% dialyzed serum and 2 mM ouabain. Some cultures also contained 50 μM bumetanide, 30 nM PMA (added 10 min before change of medium), and 0.025 mM (*A*) or 1.2 mM (*B*) Ca^{2+}. Samples of medium were collected at intervals for measurement of $^{86}Rb^+$ content. ○, No bumetanide; ●, with bumetanide; □, with bumetanide and PMA.

1.2 mM Ca^{2+}. At 40 min, cell K^+ content was less than 20% of controls. Addition of bumetanide did not significantly change the extent of K^+ loss at either low or high Ca^{2+} levels (arrow in Fig. 3 and data not shown). PMA, however, did retard K^+ loss at low Ca^{2+}. The effect of PMA (tested from
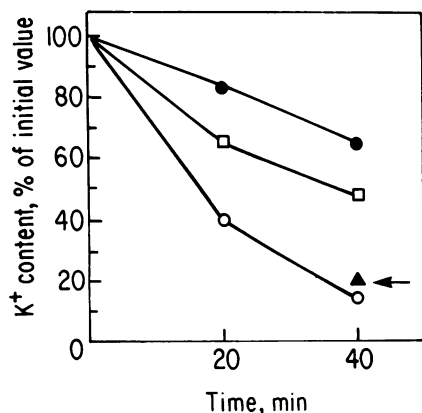

FIG. 3. K^+ efflux from NIH-3T3 cells in Ca^{2+}-deficient media. Cells in DMEM-a with 5% dialyzed serum, 2 mM ouabain, and Ca^{2+} as indicated below. Sample cultures were processed at 20 and 40 min for analysis of K^+ content. ●, 1.2 mM Ca^{2+}; ○, 0.025 mM Ca^{2+}; □, 0.025 mM Ca^{2+} with 30 nM PMA added 10 min before medium change; ▲ (data point at arrow), 0.025 mM Ca^{2+} with 50 μM bumetanide.

10 to 100 nM) was always substantial and was neither enhanced nor inhibited by the simultaneous presence of bumetanide (data not shown). At 1.2 mM Ca^{2+}, PMA produced little or no attenuation of K^+ loss (data not shown).

Phorbol esters are known to enable rat liver cells in low-Ca^{2+} medium, arrested in the G_0/G_1 phase of the cell cycle, to proceed into S phase (14). Since PMA retarded K^+ loss in Ca^{2+}-deficient medium, rescue of NIH-3T3 cells in low Ca^{2+} was attempted. PMA, without any transport inhibitors, strongly but incompletely retarded K^+ loss from NIH-3T3 cells in 0.05 mM Ca^{2+}. However, PMA (10–100 nM) did not promote growth of cells in low Ca^{2+} (data not shown), perhaps because phorbol esters rapidly desensitize cells to their receptors and to other mitogens (15).

Comparison of measurements of $^{86}Rb^+$ efflux (Fig. 2) with measurements of loss of cell K^+ determined by flame photometry (Fig. 3) reveals an important difference in the effects of bumetanide and PMA. At low Ca^{2+}, bumetanide produced a decrease in $^{86}Rb^+$ efflux but not, under similar conditions, a decrease in net loss of K^+. Hence bumetanide presumably inhibited only an exchange of intracellular $^{86}Rb^+$ (and therefore K^+) for extracellular K^+. In contrast, PMA decreased both efflux of $^{86}Rb^+$ and loss of K^+. The results suggest that PMA and Ca^{2+} inhibit K^+ efflux through a pathway that does not involve the KCl/NaCl cotransport system.

Cycloheximide Does Not Inhibit the Action of PMA. Phorbol esters are known to induce transcription of a number of messenger RNAs. To see whether synthesis of new proteins was needed for the action of PMA described above, cycloheximide—in concentrations found to inhibit protein synthesis rapidly (data not shown)—was added to NIH-3T3 cultures 2 min before PMA, and $^{86}Rb^+$ efflux was measured. PMA inhibited $^{86}Rb^+$ efflux whether or not cells were pretreated with cycloheximide (Fig. 4). Similar results were found when K^+ efflux was assessed by flame photometry (data not shown).

K^+ Efflux May Be Controlled by a Protein with a Short Half-Life. Although cycloheximide alone, if present for the relatively short period of efflux measurement, had no significant effect, longer periods of cycloheximide pretreatment produced a different result. If added to cultures 30 min or more before a change to Ca^{2+}-deficient medium, cycloheximide partially protected NIH-3T3 cells from K^+ loss. The longer the pretreatment, the greater the protection. Protec-
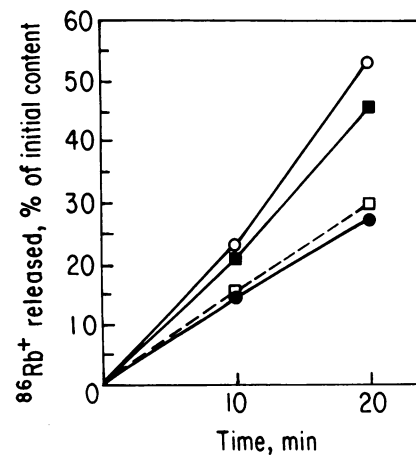

FIG. 4. Effect of cycloheximide on inhibition of $^{86}Rb^+$ efflux by PMA. $^{86}Rb^+$ was added to cultures; after 3 hr medium was changed to DMEM-a with 5% dialyzed serum, 2 mM ouabain, 50 μM bumetanide, and 0.05 mM Ca^{2+}. ○, No other additions; ■, cycloheximide (10 μg/ml) added at minus 12 min; □, PMA (30 nM) added at minus 10 min; ●, cycloheximide added at minus 12 min and PMA added at minus 10 min.

tion was substantial with 270 min of pretreatment (Fig. 5, compare bars B and F) but still not as complete as that given by briefer treatment with PMA (bars G–I). The results are consistent with the idea that a protein (or proteins) with a short half-life is needed for opening K^+ and Na^+ flux pathways when cells are placed in Ca^{2+}-deficient medium. Cycloheximide had almost no effect on K^+ efflux from NIH-3T3 cells in 1.2 mM Ca^{2+}. After cultures were briefly in low Ca^{2+}, restoration of Ca^{2+} to 1.2 mM rapidly reversed the enhancement of K^+ efflux (Fig. 5, bars B and C). In this experiment cultures were in salts/Hepes medium during incubation in low Ca^{2+}, but experiments carried out in DMEM-a gave similar results. Consequently, the components of DMEM-a that are lacking in salts/Hepes medium are not needed to elicit the actions of Ca^{2+} deficiency, PMA, and cycloheximide on K^+ efflux.

Other Agents or Conditions That Regulate K^+ Efflux. Selected channel blockers [nifedipine (10 μM) and tetraethyl-ammonium ion (2 and 4 mM)] did not retard K^+ loss. Ba^{2+} (2–5 mM) known to block some classes of K^+ channels, was tested in salts/Hepes medium and effectively inhibited K^+ loss from NIH-3T3 cells at low Ca^{2+}; high levels of Mg^{2+} (2–5 mM) also slowed K^+ loss (data not shown). Decreasing Mg^{2+} from 0.8 mM, as in DMEM-a, to 0.05 mM, with Ca^{2+} at 0.8 or 1.2 mM, did not increase K^+ efflux. Hence NIH-3T3 cells are particularly sensitive to Ca^{2+}, but other divalent cations at high levels can compensate for lack of Ca^{2+}. Puromycin (10 μg/ml) produced the same results as did cycloheximide, and 4β- (but not 4α-) phorbol 12,13-didecanoate (30 nM) produced the same results as PMA (data not shown).

Ca^{2+}-Dependent K^+ Efflux in Other Types of Untransformed Cells. The increase in K^+ efflux in low-Ca^{2+} medium described for NIH-3T3 cells was also found in human foreskin and lung (MRC-5) fibroblasts. At 0.01 mM Ca^{2+}, PMA (30 nM) inhibited bumetanide-resistant $^{86}Rb^+$ efflux by 30–35%; even at 1.2 or 1.6 mM Ca^{2+}, PMA consistently inhibited efflux by 20–30%. Measurements by flame photometry of K^+ efflux gave results similar to those of $^{86}Rb^+$ efflux (data not shown). Subconfluent cultures of Swiss 3T3a cells were relatively insensitive to low Ca^{2+}; Ca^{2+} had to be reduced to 0.01 mM to show enhanced K^+ efflux clearly (data not shown).
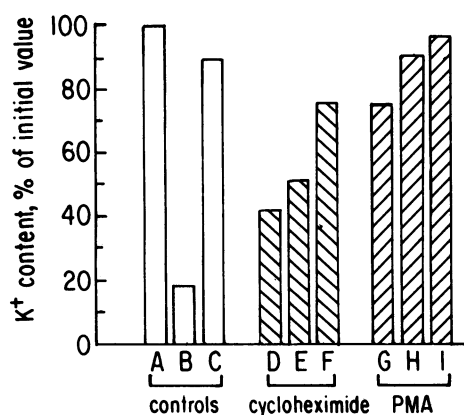

FIG. 5. K^+ efflux in Ca^{2+}-deficient medium: Effect of length of pretreatment with cycloheximide or PMA. NIH-3T3 cells were in salts/Hepes medium with 5% dialyzed serum. All cultures contained 2 mM ouabain, 50 μM bumetanide, and Ca^{2+} as indicated below. Cycloheximide (10 μg/ml) and PMA (30 nM) were added before medium change, at times shown below. Cultures were processed at 45 min for analysis of K^+ content, shown as percent of control (bar A). Bars: A, 1.2 mM Ca^{2+}; B, 0.01 mM Ca^{2+}; C, 0.01 mM Ca^{2+} for 5 min, then raised to 1.2 mM; D–F, 0.01 mM Ca^{2+}, cycloheximide added at minus 30, 90, or 270 min; G–I, 0.01 mM Ca^{2+}, PMA added at minus 10, 30, or 90 min.

K^+ Efflux from Transformed Derivatives of NIH-3T3 Cells. The two transformed NIH-3T3 cell lines studied, SV-NIH-3T3 and mT-NIH-3T3, when in 10% serum-supplemented DMEM-a with 0.05 mM Ca^{2+}, maintained high levels of cell K^+ and grew rapidly. In contrast, NIH-3T3 cells did not grow in the same medium. Therefore, it was of interest to measure K^+-efflux rates in the transformed lines at low Ca^{2+} levels.

At 0.025 mM Ca^{2+}, a level that produces a marked increase in K^+ efflux in NIH-3T3 cells (Table 1 and Fig. 3), efflux rates from the two transformed derivatives were only slightly enhanced (for SV-NIH-3T3, Fig. 6A) or were unchanged (for mT-NIH-3T3, Fig. 6B). The average decrease in K^+ content of SV-NIH-3T3 cells in 20 min, in four experiments, was 16.8 ± 3.8% (mean ± SEM) in 1.2 mM Ca^{2+} and 25.7 ± 5.3% in 0.025 mM Ca^{2+}; pretreatment of SV-NIH-3T3 cells in low Ca^{2+} with PMA or cycloheximide had little effect on K^+ efflux (Fig. 6 and data not shown). The mT-NIH-3T3 cells were insensitive to both agents (data not shown). Measurement of the bumetanide-resistant fraction of $^{86}Rb^+$ efflux in the two transformed cell lines showed a similar relative insensitivity to low Ca^{2+} levels and to PMA. Measurements of K^+ influx in SV-NIH-3T3 cells showed a small increase (ranging from 15% to 38% in three experiments) in 0.025 mM Ca^{2+}, compared to controls in 1.2 mM Ca^{2+} (data not shown); hence any modest increase in efflux could be balanced by increased influx.

DISCUSSION

NIH-3T3 cells in Ca^{2+}-deficient medium show increased K^+ efflux. This increase is inhibited by PMA and by cycloheximide. A hypothesis based on these results is proposed:
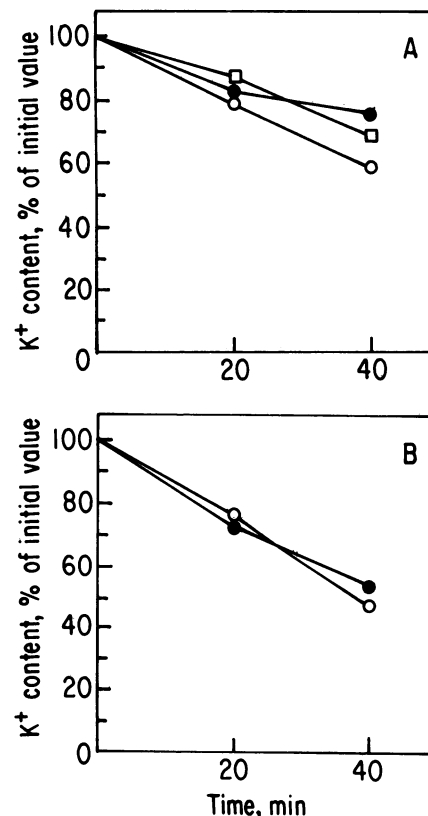

FIG. 6. K^+ efflux from SV-NIH-3T3 (A) and mT-NIH-3T3 (B) cells in Ca^{2+}-deficient medium. Cells were in DMEM-a with 5% dialyzed serum, 2 mM ouabain, and Ca^{2+} as indicated below. Sample cultures were processed at 20 and 40 min for analysis of K^+ content. ●, 1.2 mM Ca^{2+}; ○, 0.025 mM Ca^{2+}; □, 0.025 mM Ca^{2+} with 30 nM PMA added 10 min before medium change.

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enhancement of K^+ efflux (and Na^+ influx) involves a pathway or channel that is, or can become, nonselective for these ions. Flux through this channel is presumably under the control of protein kinase C and other proteins. Human fibroblasts also display increased K^+ efflux in Ca^{2+}-deficient medium. Even in 1.6 mM Ca^{2+}, higher than the level of ionized Ca^{2+} (1.2 mM) in human plasma (16), these cells show some inhibition of K^+ efflux by phorbol esters, and this finding suggests that protein kinase C regulates K^+ efflux under physiological conditions.

In a previous study (6), enhanced K^+ efflux was observed in a medium deficient in Ca^{2+} and containing only 0.1 mM K^+. In the present study, when such low levels of K^+ were present in Ca^{2+}-deficient media, bumetanide, as well as PMA, inhibited K^+ efflux (data not shown), and an unambiguous separation of the effects of PMA from those of bumetanide could not be made. Hence the decision to study K^+ efflux at about 5 mM K^+ (the physiological level) made it possible to identify a component that is insensitive to bumetanide but is inhibited by PMA.

When the level of Ca^{2+} in the medium, normally above 1 mM, is lowered to 0.05 mM, the rate of K^+ efflux exceeds the capacity of the Na^+,K^+-ATPase pump to move K^+ back into the cell. Growth rate declines. Since cell K^+ levels are known to control protein synthesis and response to mitogens (17–21), changes in cell K^+ and Na^+ seem sufficient to explain decreased growth in Ca^{2+}-deficient media. Other Ca^{2+}-dependent processes, however, may be inhibited in Ca^{2+}-deficient media and thus account for growth inhibition (22). Hence the contribution of changes in cell K^+ to growth retardation in low-Ca^{2+} media is not settled.

In the mouse and human fibroblasts studied here, growth rate increases progressively as the level of Ca^{2+} in the medium is raised, until a plateau is reached at about 0.2 mM Ca^{2+} (data not shown). Keratinocytes, however, show a different pattern of response to Ca^{2+}: growth rate is optimal at 0.05–0.1 mM Ca^{2+}. At higher levels, growth slows or stops and the cells differentiate; at very low levels of Ca^{2+}, growth also ceases (23). Hence keratinocytes, like fibroblasts, do show a minimum Ca^{2+} requirement for growth.

Nonselective movement of monovalent cations through Ca^{2+} and K^+ channels, enhanced at low Ca^{2+}, has been observed in patch-clamp and other studies with a variety of cell types including heart and skeletal muscle, squid neuron, and toad bladder (24–27). It seems plausible that NIH-3T3 cells and human fibroblasts have channels that display some loss of selectivity for cations.

The results in this paper offer an explanation for Ca^{2+} dependence, as displayed by NIH-3T3 cells and human fibroblasts, and relative Ca^{2+} independence, described here for two transformed cell lines. The transformed cell lines show little or no increase in the rate of K^+ efflux when in low-Ca^{2+} media, and hence they can maintain normal levels of intracellular K^+. It is premature to suggest, however, that transformation is often linked to a change in K^+-efflux properties. Further studies on a variety of isogenic pairs of untransformed and transformed cells are needed.

The rate of K^+ efflux in cultured cells is surprisingly high—about 1% of K^+ content per minute. This efflux, which must be balanced by action of the pump, puts a metabolic demand on the cell and seems to be subject to control. Hence the K^+ efflux pathway (or channel) hypothesized above may turn out to have important functions in the control of cell metabolism, growth, and differentiation.

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