Correlation of ouabain-sensitive ion movements with cell-cycle activation

(Na⁺,K⁺-ATPase/dihydrotelocidin B/phorbol 12-myristate 13-acetate)

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ABSTRACT The role of tumor-promoter-induced Na⁺, K⁺-ATPase activity in cell proliferation and the extent of coupling of Na⁺ and K⁺ movements to cell-cycle control under differing physiological states was examined. Earlier studies indicated that staging of cells in G₁ by serum deprivation in the presence of phorbol esters such as phorbol 12-myristate 13-acetate (PMA) induced a state(s) in which postconfluent C3H 10T½ fibroblasts were refractory to ouabain inhibition of DNA synthesis, and the present study further examines this property. Previous findings suggested that the promoter can act in either of two ways: (i) it can act by inducing an alternative pathway to S-phase independent of Na⁺,K⁺-ATPase activity, or (ii) the promoter can advance the cells in G₁ to a point beyond which Na⁺,K⁺-ATPase activity is no longer required for the induction of DNA synthesis. When ouabain (0.3 mM) was added simultaneously with tumor promoters, such as dihydrotelocidin B, to cells arrested in the G₁ phase, [³H]thymidine incorporation was inhibited >90%. These data suggest that an alternative pathway is not likely the explanation but that tumor promoters advance cells through a dynamic state in G₁, during which progression toward S-phase entry is independent of a Na⁺,K⁺-ATPase dependent regulatory step. Ouabain sensitivity kinetics measured by two independent methods indicated that the development of ouabain insensitivity is found in G₁ > 2 hr prior to S phase. This study indicates that the measured ion movements are markedly dependent on cell-cycle state and describes the criteria required to obtain reproducible responses.

As pointed out by Pardee and co-workers (1, 2), as cells pass through G₁ they must undergo a number of biosynthetic processes that together constitute the elements of a regulatory system for cell proliferation. It is probable that the G₁ phase of the cell cycle does not represent a unique G₁ state but rather several substates. Baserga (3) has noted that specific qualitative differences are needed which correspond to specific G₁ substates analogous to DNA synthesis in the S phase. Recent studies indicate that cells in G₁ can be categorized into states with different biochemical characteristics and that these states may differ in terms of regulation of metabolic processes. Wille and Scott (4) describe physiological states induced by amino acid deprivation or serum withdrawal that differ from those associated with initiation of differentiation processes. Further evidence has been obtained that cell cycle control of c-myc but not c-ras expression in fibroblasts is lost after transformation, and expression of the myc and ras proto-ondences is related to cell-cycle timing (5). When fibroblasts were exposed to phorbol ester tumor promoters, specific substates in G₁ could be demonstrated (6). Furthermore, Jones et al. (7) have demonstrated that with Ca²⁺ deprivation, a population of cells are blocked at a point 2 hr prior to S-phase entry. Reinitiation by tumor promoters results in a bypass of the Ca²⁺ requirement so that S-phase entry proceeds within 2 hr of the administration of tumor-promoting phorbol esters. Cells blocked at this point have specific properties that may be associated with particular ion transport responses, and it would be useful to have markers based on ion transport to distinguish substrates in G₁.

We consider that Na⁺,K⁺-ATPase activity may serve as a marker for cells within the G₁ state, because the pump is elevated after cell-cycle activation. Quastel and Kaplan (8) first reported ouabain-sensitive DNA synthesis and that an increased uptake of K⁺ ions was necessary for the biosynthetic events of lymphocyte activation. Also, proliferation of cultured Ehrlich ascites cells was reported to be depressed through inhibition of cation transport by ouabain (9). Recently, it was demonstrated that staging of cells in G₁ by serum deprivation in the presence of phorbol esters such as phorbol 12-myristate 13-acetate (PMA) produced a population of cells that undergoes DNA synthesis in the presence of ouabain (10). It would appear that ouabain might also be used to characterize a specific G₁ state that has defined biochemical characteristics.

Our findings of a tumor-promoter-induced ouabain-insensitive DNA synthesis could be interpreted in several ways: (i) PMA and dihydrotelocidin B (DHTB) could act by inducing an alternative pathway to S phase independent of Na⁺,K⁺-ATPase; (ii) the promoter can advance the cells to a point at which Na⁺,K⁺-ATPase is no longer required for the induction of DNA synthesis; (iii) the promoter induces a ouabain-insensitive Na⁺,K⁺-ATPase. If it can be shown that the staging procedure advances cells in G₁ to a state in which DNA synthesis is refractory to ouabain inhibition, then it would be possible to obtain an additional parameter to define a substrate in G₁. In the current study, we examined the role of tumor-promoter-induced Na⁺,K⁺-ATPase activity in C3H 10T½ fibroblast proliferation and the extent by which Na⁺ and K⁺ movements are coupled to cell-cycle control under different physiological states. There is particular emphasis on the necessity for kinetic studies throughout the cell cycle that characterize the sensitivity to ouabain at different time points.

MATERIALS AND METHODS

Cultures of C3H 10T½ mouse fibroblasts (passages 7-14) were maintained as described (6) using basal Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Sterile Systems, Logan, UT). Experiments were performed on duplicate cultures in 60-mm-diameter culture plates seeded in 5-ml aliquots at 10⁶ cells per plate. Growth medium was renewed every 3-4 days. Confluence was reached at

Abbreviations: PMA, phorbol 12-myristate 13-acetate; DHTB, dihydrotelocidin B.

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8-10 days, and postconfluent cells were used (48-72 hr after the time of confluence).

Cell-cycle activation of C3H 10T½ mouse fibroblasts was evaluated in either of two ways: (i) with cells arrested in G1 by density-dependent inhibition (i.e., basal Eagle’s medium and 10% fetal calf serum that was added 48-72 hr prior to introduction of tumor promoter) or (ii) cells were staged in G1 by serum deprivation in the presence or absence of a tumor promoter as described (6).

The first procedure provides a measure of direct cell-cycle activation because these cells are found to be fully quiescent when postconfluent cells are used. At the designated time intervals, [3H]thymidine (0.2 μCi/ml; 37 Ci/mmole; 1 Ci = 37 GBq) was introduced for 1 hr for pulse incorporation or for the indicated times after PMA or DHTB introduction at t = 0 for cumulative measurements. [3H]Thymidine concentrations were added at 5 nM in order to avoid alterations associated with uptake and nucleotide pool sizes (11).

The reaction was stopped by removal of medium containing [3H]thymidine followed by the addition of unlabelled 1.5% perchloric acid. [3H]Thymidine incorporation into the acid-insoluble fraction was assayed as described (6).

In the second procedure, cells were staged in medium with low serum (0.5% fetal calf serum) and tumor promoters for 24 hr and then reinitiated with 10% fetal calf serum and basal Eagle’s medium. [3H]Thymidine incorporation was measured as described above and cell numbers were obtained with a Coulter electronic cell counter.

Uptake studies were carried out with pre- or postconfluent cells similarly to that reported earlier (12). 86Rb⁺ uptake studies were initiated in duplicate culture plates by addition of 2 μCi of 86Rb per plate. After incubation, the supernatant was removed and the cells were quickly washed once with phosphate-buffered saline (room temperature). Trichloroacetic acid (5%) was then added to release intracellular 86Rb⁺, followed by two washes, and 86Rb⁺ was then measured. Studies were carried out in the presence and absence of ouabain as a measure of uptake mediated by Na⁺,K⁺-ATPase.

Materials. DHTB was a kind gift of T. Sugimura and H. Fujita (National Cancer Center Research Institute, Tokyo). PMA was obtained from Chemical Carcinogens, Eden Prairie, MN. 86RbCl was obtained from Nuclear Sciences and Technology, Buffalo, NY. [3H]Thymidine was obtained from ICN. Basal Eagle’s medium was from GIBCO.

RESULTS

Correlation Between Na⁺,K⁺-ATPase Activity and DNA Synthesis by C3H 10T½ Cells in Density-Dependent Arrested Cells. Previous studies have indicated that tumor promoters exhibit both stimulatory and inhibitory responses on ion fluxes mediated by Na⁺,K⁺-ATPase (13-15). These contradictory results indicate the necessity for study of the kinetics of ion movements under conditions of defined cell-cycle states. Cells that are fully quiescent are used to avoid heterogeneous cell-cycle states. To obtain fully quiescent postconfluent cells, we find it is necessary to wait 48-72 hr after a medium change, a time at which DNA synthesis is minimal and a time at which growth factors are believed to be depleted. Under these conditions, [3H]thymidine incorporation has a basal level of <2000 cpn per culture plate throughout the time period under study (Fig. 1). When DHTB (10 nM) is added directly to these quiescent cells 72 hr after medium change, there is a peak stimulation of 1-hr pulse [3H]thymidine incorporation about 25 hr later. Little or no change in [3H]thymidine incorporation is observed when ouabain (1 mM) is added at the time of the pulse.

As shown in Fig. 1 (Lower), 86Rb⁺ uptake was also measured simultaneously for 1-hr periods in replicate cultures. When DHTB was added to cultures at t = 0 and 86Rb⁺ uptake was measured during the subsequent hour, the response was a 15% stimulation relative to the control. The development of a more pronounced 86Rb⁺ uptake is progressive with time after DHTB addition, and as shown, this markedly increasing rate of uptake is ouabain sensitive, indicative of Na⁺,K⁺-ATPase activity.

When cells are fed 24 hr prior to the introduction of DHTB (Fig. 2), the responses differ from those fed 72 hr prior to the introduction of DHTB (Fig. 1). It is logical to assume that the cells that had responded with enhanced DNA synthesis to serum addition 24 hr previously are likely to be in the later stages of cell cycle, as indicated by the downward slope of [3H]thymidine incorporation. With DHTB addition to these cells at t = 0, a significant suppression of [3H]thymidine incorporation was noted at 8 hr. Nevertheless, a new cycle of DNA synthesis is initiated indicative of continued cell-cycle progression. As seen in Fig. 2, at t = 0, DHTB initially inhibits the rate of 86Rb⁺ uptake measured after 1 hr of incubation. However, the pattern that emerges with time is an increase in ouabain-sensitive 86Rb⁺ uptake stimulated by DHTB when compared to control. The observed increase in
cells 24 hr after medium replicate 

[$^{3}$H]thymidine incorporation by medium 24 Eagle's 86Rb+ uptake reveal that the ion state, obtained. This enter S 10% fresh studies indicated that incorporation by 33%, to incorporation results indicated ouabain previously reported (10). [$^{3}$H]thymidine in FIG. 2.

Figs. Ability examined both with and without simultaneous addition of ouabain. In each case, similar responses were obtained. This supports the concept that at the time cells enter S phase, they are refractory to ouabain inhibition.

Ability of DHTB Staging to Perturb Serum-Induced Ouabain-Insensitive [$^{3}$H]thymidine Incorporation. Earlier studies indicated that treatment of cells with PMA in serum-free medium produced a ouabain-insensitive [$^{3}$H]thymidine cumulative incorporation upon reinitiation of cell cycle with fresh 10% fetal calf serum (10). This observation has been extended to DHTB (10 nM)-pretreated cultures, and the results indicated ouabain only inhibited [$^{3}$H]thymidine incorporation by 33%, which is similar to the effects of PMA previously reported (10). In contrast, in untreated cells staged in low serum, ouabain (1 mM) inhibited serum-initiated [$^{3}$H]thymidine incorporation by 89%, confirming

$^{86}$Rb+ uptake when DHTB is added in combination with ouabain is reproducible but unexplained. These studies reveal that the ion transport responses are complex unless cell-cycle states are defined. If cells are not in a synchronous state, the results are difficult to interpret.

In Figs. 1 and 2, pulse [$^{3}$H]thymidine incorporation kinetics were examined both with and without simultaneous addition of ouabain. In each case, similar responses were obtained. This supports the concept that at the time cells enter S phase, they are refractory to ouabain inhibition.

Fig. 3. Effect of ouabain added simultaneously with DHTB and [$^{3}$H]thymidine pulse incorporation was measured at various time intervals. Postconfluent cells that were density-dependent-arrested (72 hr after medium change) were treated with DHTB (10 nM) (a), ouabain (2.7 × 0.1 nM) (c), DHTB and ouabain (d), or no additions (b) (control). Pulse [$^{3}$H]thymidine incorporation into acid-insoluble material was measured as a function of time of pulse addition that Na+K+-ATPase activity is essential for serum-induced DNA synthesis (13). Regardless of whether DNA synthesis was stimulated by tumor promoters or serum, the data indicate that cells pass through a transitional state that is refractory to ouabain inhibition.

Does The Tumor Promoter Act by Inducing an Alternative Pathway to S Phase Independent of the Na+K+-ATPase Activity? The effect of ouabain on DHTB-induced [$^{3}$H]thymidine incorporation was then examined with density-inhibited cells arrested in the G1 phase (72 hr after medium change). As described in Fig. 3, 10 nM DHTB was added with or without simultaneous addition of ouabain to postconfluent cells 48 hr after renewal of growth medium. Pulse incorporation of [$^{3}$H]thymidine (1 hr) was then measured at various time intervals. DHTB stimulated [$^{3}$H]thymidine incorporation at 12–18 hr, which is similar to that observed with serum stimulation. [$^{3}$H]thymidine incorporation was inhibited >90% when ouabain was added simultaneously with DHTB. These data reveal that DHTB-induced DNA synthesis is ouabain sensitive. Apparently, DHTB can advance the cells to a point that is independent of a Na+K+-ATPase pump, rather than bypassing a ouabain-sensitive stage, because addition of ouabain up until 12 hr after DHTB addition will not achieve a ouabain-insensitive S-phase entry (13).

The Determination of the Time Frame in the Cell Cycle at Which the Ouabain-Insensitive DNA Synthesis Develops. A further step was taken to determine the time frame in which a ouabain-insensitive state exists. Two separate types of experimental procedures were performed to achieve G1 arrest. The first approach was with postconfluent cells (72 hr after medium change) (Fig. 4 a and b); the second was with preconfluent cells that had been staged in low serum with and without 10 nM DHTB (Fig. 4 c and d).

In the first approach described in an experiment (Fig. 4 a and b), postconfluent quiescent cells were treated at t = 0 with DHTB to activate a synchronous population of cells moving through G1–S phase. Pulse [$^{3}$H]thymidine incorporation was examined at various time intervals to monitor cell progression; using duplicate cultures, cumulative DHTB-induced [$^{3}$H]thymidine incorporation was measured as a function of the time at which ouabain (1 mM) was added. It became apparent that cells that are in the ouabain-insensitive
state will progress in G1 to the S phase, whereas cells that have not reached this state will be prevented from entering the DNA synthesis period. As shown in Fig. 4a and b, the development of ouabain insensitivity occurred 12 hr after the initiation by DHTB and had a t\textsubscript{1/2} (time at which 50% of ouabain-insensitive \textsuperscript{3}H]thymidine incorporation was observed) of 17 hr. Pulse incorporation kinetics indicated a t\textsubscript{1/2} (time at which 50% of the maximum peak incorporation of \textsuperscript{3}H]thymidine was observed) at 19 hr. Therefore, the development of the ouabain-insensitive state occurs approximately 2 hr prior to S-phase entry.

In the experiment described in Fig. 4c and d with preconfluent cells, after staging in low serum in the presence of DHTB, duplicate cultures were divided into two groups: one group to determine ouabain sensitivity through cumulative \textsuperscript{3}H]thymidine incorporation measurements at the designated time periods, and the other to determine S-phase entry kinetics. After a 48-hr DHTB staging, a time at which cells are arrested in G1, cells are reinitiated at t = 0 by replacing medium with basal Eagle’s medium/10% fetal calf serum. The decrease in sensitivity to 1 mM ouabain with time was measured after cell-cycle activation with serum and was then compared with S-phase entry kinetics. It can be seen that when ouabain was added at t = 0, 24-hr cumulative \textsuperscript{3}H]thymidine incorporation was depressed 52%. This inhibition decreased with time and the t\textsubscript{1/2} was calculated to be 9.5 hr. Pulse incorporation of \textsuperscript{3}H]thymidine revealed that the t\textsubscript{1/2} for S-phase entry was 11.5 hr, 2 hr later than that obtained for ouabain-sensitivity kinetics. The control pulse incorporation of \textsuperscript{3}H]thymidine was found to have a t\textsubscript{1/2} of 13.5 hr, 2 hr later than the DHTB staged cells, which is in accord with previous findings using PMA (6). Therefore, this evidence supports the existence of a ouabain-insensitive state which is within 2 hr of S-phase entry, in accord with the point at which the ouabain-insensitive state had developed in the experiment described in Fig. 4a and b with postconfluent cells.

**DISCUSSION**

Biochemical activities such as ion transport processes are tightly controlled events that play an important role in the regulation of the cell cycle. Divergent responses obtained in different laboratories show a need for proper control of experimental parameters. The present study demonstrates that the ability to observe direct tumor-promoter-induced K\textsuperscript+ or Rb\textsuperscript+ uptake mediated by the Na\textsuperscript+,K\textsuperscript+-ATPase critical to cell-cycle activation is reproducible, provided that the following experimental conditions are maintained: (i) Cells must be in the G1 phase of the cell cycle, because optimal \textsuperscript{86}Rb\textsuperscript+ uptake is observed when cells are just prior to or at the point of S-phase entry. (ii) The time of the last medium
change must be rigidly adhered to (between 48 and 72 hr under our conditions). (iii) Appropriate cell-response kinetics are necessary to understand changes in cell-cycle activation. The studies of responses at single time periods can be misleading and do not provide an adequate basis for proposing specific mechanisms involved.

A pertinent finding of this study is the reproducible stimulation of $^{86}$Rb$^+$ uptake after DHTB administration. This stimulation of $^{86}$Rb$^+$ is observed during the first hour of incubation, but it is also noted to increase with time. The progressively increasing ouabain-sensitive $^{86}$Rb$^+$ uptake may indicate a threshold level of Na$^+$,K$^+$.ATPase activity at about the time the majority of cells enter S phase.

We have observed both stimulation and inhibition of $^{86}$Rb$^+$ uptake by tumor promoters depending on the change in parameters as stated above (cf. Figs. 1 and 2). Failure to show enhancement of K$^+$ influx by tumor promoters (15) is possible if cells are distributed throughout different phases of the cell cycle. If one considers only one time point, it is possible to conclude that tumor promoters only inhibit K$^+$ uptake.

It is apparent that DHTB has the ability to stimulate a population of cells to move synchronously through G$_1$ into the S phase of the cell cycle. This is indicative of a direct mitogenic action, and our evidence supports the idea that DHTB is not just a co-mitogen but that it can act without subsequent addition of other cofactors. DHTB can markedly amplify the number of cells entering S phase compared with either serum or epidermal growth factor (data not shown). The data show that DHTB does not simply bypass the requirement of Na$^+$,K$^+$.ATPase activity for advancement in cell cycle, nor does it induce a ouabain-insensitive Na$^+$,K$^+$.ATPase activity, but DHTB initiates cells to advance to a state that is no longer sensitive to ouabain.

The experimental evidence supports the development of a transient ouabain-insensitive state in G$_1$ that is approximately 2 hr prior to S-phase entry. This was demonstrated in both preconfluent and postconfluent quiescent cells by two independent staging techniques. In postconfluent quiescent cells, the time at which 50% of the cells reach peak [H]$^+$thymidine incorporation is shown to be 19 hr; the development of 50% of the ouabain-insensitive cells entering S phase was found to be 17 hr. Similarly, in preconfluent cells that had been staged with DHTB in low serum, the time at which 50% of peak pulse incorporation occurs is shown to be 11 hr. The time point at which ouabain can inhibit 50% of cumulative DNA synthesis was found to be 9 hr. Thus, in each case, the development of a ouabain-insensitive state is found to be in G$_1$ = 2 hr prior to S-phase entry.

The question that now arises is why the Na$^+$,K$^+$.ATPase activity is required just prior to S-phase entry. One may consider the following possibilities: (i) The ionic environment has a critical regulatory role in translational and/or transcriptional processes (e.g., changes in ionic environment have been shown to influence B to Z DNA transitions, which are presumed to play important roles in transcriptional control (16)). Also, critical K$^+$ changes have been shown to be essential for peptide bond synthesis in forward translation processes (17)—e.g., synthesis of a restriction protein. It is also possible that ion movements are critical for a backward translation process such as in the synthesis of Ap$_4$A, a nucleotide elevated just prior to S-phase entry (cf. ref. 18). (ii) Na$^+$,K$^+$.ATPase activity has been implicated in volume control; cells may need to reach a volume threshold level before S-phase entry can proceed (19). (iii) Na$^+$,K$^+$.ATPase activity has been shown to be tightly coupled to the glycolysis pathway; therefore, the pump's activity may be necessary for maintaining the bioenergetics of the cell. (iv) The ionic environment has a critical regulatory role on an enzyme whose activity is needed for advancement of cells through the cell cycle. Interruption of any of these processes could lead to failure of the cell to advance in the cell cycle.

We do not wish to imply that the observed cation-dependent events are specific for tumor promoters or that the bypass of Na$^+$,K$^+$.ATPase dependency represents a specific relationship to tumor promotion. However, we consider that cells exist in both stable and dynamic physiological states in G$_1$ and that our understanding of states in G$_1$ and their relationship to growth regulations is worthy of further study. It is likely that other mitogenic agents behave similarly and that tumor promoters represent a useful tool to study growth regulatory mechanisms. It is our belief that transitions between cell-cycle states are controlled by changes in membrane processes and their associated ion movements.

The present study emphasizes that cells can be arrested in different points in G$_1$, and the use of different G$_1$ markers may help to elucidate the mechanism by which various mitogenic agents act. Staging techniques such as those described here to characterize Na$^+$,K$^+$.ATPase activities in G$_1$ with concomitant study of reactions involving assembly of critical components necessary for DNA synthesis may be useful to further probe the state(s) in G$_1$ that monitors the cells' ability to advance in the cycle.

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