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An early transient increase of intracellular Na^+ may be one of the first components of the mitogenic signal. Direct detection by ^{23}Na-NMR spectroscopy in quiescent 3T3 mouse fibroblasts stimulated by growth factors

Elisha Berman *, Ilana Sharon, Henri Atlan

Human Biology Research Center / Department of Medical Biophysics, Hadassah University Hospital, Jerusalem, IL-91 120, Israel

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Abstract

^{23}Na-NMR spectroscopy was designed to allow for continuous recording of intracellular Na^+ in 3T3 fibroblasts stimulated by serum growth-factors in the presence of ion transport inhibitors. The metabolic state of cells at rest and following stimulation was monitored by ^{31}P-NMR spectra of ATP and related high-energy phosphates. The study demonstrates that early activation of ion transporters by addition of serum is marked by the appearance of transient increase of the intracellular Na^+, beginning 3 min after addition of serum to quiescent culture and lasting approx. 20 min. The initial rise in cellular Na^+ results from an increased activity of the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport and of the amiloride-sensitive Na^+/H^+ antiport. It is suppressed by any one of these inhibitors. Subsequent activation of the ouabain-sensitive Na^+/K^+-ATPase results in an increased Na^+ efflux, leading to a return of intracellular Na^+ to its initial baseline. Previous work had shown that the early activation of bumetanide-sensitive and amiloride sensitive ion-transporters by growth-factors was essential for induction of cell division, at least in some cell types. Preventing ion activation by adding ion-transport inhibitors lead to the inhibition of DNA synthesis 18 h later. This process was reversible upon elimination of these inhibitors. Even though alternative non-specific effects of these inhibitors cannot be ruled out, the observed transient peak in intracellular Na^+ may be one of the earliest components of the mitogenic signal. On the basis of previous works, its effect seems to be related to the activation of Ca^{2+}-dependent and cyclic AMP second messenger pathways. The different mechanisms whereby the activated $Na^+/K^+/Cl^-$ cotransport and the Na^+/H^+ antiport contribute to this signal need to be further investigated.

Keywords: Sodium ion, intracellular; Intracellular sodium ion; NMR; Ion flux; Cell culture; Mitogenic signal; (3T3 fibroplast)

1. Introduction

Changes in cell membrane permeability play a key role in the fundamental processes of cell growth and mitogenesis. One of the earliest responses of quiescent cells to a mitogenic stimulus is cation fluxes activation [1–3]. Two sodium transport systems were shown to be stimulated by growth-factors: the amiloride-sensitive Na^+/H^+ antiport [4–7], and the bumetanide-sensitive $Na^+/K^+/Cl^-$ co-transport [8–14]. When either bumetanide or amiloride is added to quiescent cells, it partially inhibits the observed increase in the intracellular Na^+ that follows the addition of serum to BALB/c mouse 3T3 fibroblasts [14]. In the presence of both amiloride and bumetanide, a complete inhibition of the early increase in the intracellular Na^+ was demonstrated [15]. Inhibition of Na^+ fluxes by amiloride or by bumetanide was also demonstrated in human skin fibroblasts and in endothelial cells [16,17]. Moreover, the bumetanide did produce inhibition of DNA synthesis that was reversed after the removal of the inhibitor [16,17]. The activating role of these ion transporters as part of the mitogenic signal has been studied in conjunction with the activation of Ca^{2+}-dependent second messenger pathways [16,18]. In all of these studies, the cellular Na^+ content was difficult to measure because of its relatively low concentration compared to its extracellular concentration. In addition, the role of those ion pathways in mitogenesis has been questioned due to the variability of the results

* Corresponding author. Fax: +972 2 438803.

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obtained in different cell types [19] and to the possibility of nonspecific basic effects of the inhibitors, e.g., the documented inhibition of protein kinases by amiloride at higher concentrations [15,20]. It is difficult to answer these questions in the absence of additional data on the ionic changes themselves that would provide testable elements for plausible mechanism. The purpose of this study was to follow more precisely the time course of sodium cell content following mitogenic stimulation and to obtain information on, at least, some of its regulatory mechanisms.

Traditionally, the modifications of cation fluxes and concentrations have been investigated using numerous classical and conventional methods that often employ various radioactive tracers. These methods include stages in which the cells are washed by non-physiological fluids resulting in possible introduction of unwanted artifacts in the measurements. The greatest advantage of nuclear magnetic resonance (NMR) spectroscopy as the method of choice, lies in its ability to perform non-invasive measurements in real time under full physiological conditions [21]. Cell cultures are routinely introduced into the NMR magnet; they are kept well thermostated and oxygenated for significant time spans without unwanted side effects [22]. The setup of the perfusion system lends itself to easy administration of drugs and other biological precursors ([23] and references cited therein). Sodium NMR (^{23}Na-NMR) spectroscopy has been employed in the study of sodium fluxes in various cell cultures [24,25]. In a conventional ^{23}Na-NMR experiment, paramagnetic chemical-shift reagents (SRs) are used to distinguish between resonances corresponding to the intracellular and the extracellular parts of the Na^+ pool. The SR interacts only with the extracellular Na^+, producing a selective spectral shift of the corresponding ^{23}Na-NMR signal. Thus, SRs provide a direct mean of detecting the intracellular Na^+ signal in the presence of the 10-fold larger extracellular Na^+.

Activation of the Na^+ transporters by growth-factors can lead to either a permanent or a transient increase in the intracellular Na^+ pool. The former case would mean that the baseline intracellular Na^+ pool is higher in proliferating cells than in quiescent cells. Since the published studies did not satisfactorily resolve this issue, we have designed and initiated a study that would provide clearer answers. Using ^{23}Na-NMR spectroscopy, we observed sodium flux changes in cell culture kept under in vivo conditions.

Our study is the first one to demonstrate the occurrence of a transient rather than a permanent increase in the intracellular Na^+ following addition of serum to quiescent cells. We show that amiloride and bumetanide inhibit the Na^+ influx through the Na^+/H^+ antiport and the $Na^+/K^+/Cl^-$ cotransport respectively, and completely suppress the transient Na^+ signal in the cells. In the presence of ouabain, the intracellular Na^+ signal completely lost its transient nature.

2. Materials and methods

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Biological Industries, Beit-Haemek, Israel. Amiloride-hydrochloride, bumetanide, ouabain and both the dysprosium-chloride-hexahydrate and the pentasodium tripolyphosphate used in the preparation of dysprosium polyphosphate complex, $Dy(ppp)_2^{7-}$, were purchased from Sigma, St. Louis, MO, USA. The Biosilon* micro-carrier beads were ordered from A/S NUNC, Roskilde, Denmark. We prepared stock solutions of bumetanide (2 mg/ml), amiloride (150 mg/ml) and ouabain (270 mg/ml) in DMSO and kept them refrigerated in the dark to prevent chemical deterioration. Freshly prepared 100 mM stock solution of $Dy(ppp)_2^{7-}$ in buffer was used for the sodium flux studies.

2.1. Cell culture preparation

We maintained BALB/c 3T3 fibroblasts in serum-rich (10% FCS) DMEM under humidified atmosphere of 10% CO_2 at 37°C. The seeded cells were placed in bacteriological Petri dishes together with micro-beads ($\approx$ 2000 cells/mg of wet weight of beads) [23]; they allowed to grow to near confluence (5–7 days). At that stage, the cells' medium was replaced by a medium containing only 5% FCS to avoid possible cell detachment. After 24 h in that medium, the beads were pooled (1.5–2 ml, containing $\approx 2 \cdot 10^7$ cells) and loaded into the NMR glass tube. The packed beads were perfused continuously (1.1 ml/min) with well oxygenated (95:5 O_2/CO_2) serum-deficient (0.5% FCS) DMEM. A complete quiescent cell culture was obtained 18–20 h after placing it in the NMR magnet.

2.2. NMR experiments

We recorded the 162 MHz ^{31}P-NMR and the 105.8 MHz ^{23}Na-NMR spectra on a Bruker AMX400WB spectrometer equipped with a 20 mm multi-nuclear probe head. The probe head's temperature was maintained at 37°C using the temperature control unit supplied with the instrument. Plastic tubing to and from the medium's reservoir was connected to the 10 mm NMR sample tube from the top of the magnet. Measured amounts of the prepared stock solutions were introduced through a side arm in the reservoir container at respective final concentrations given in the text (see below).

^{31}P spectra

Spectra were recorded with a spectral window of 9090 Hz using 8192 data points, 45° RF pulse (14 μs) and 4000 transients spaced one second apart. The time domain data was zero-filled to 16384 data points and transformed with a 20 Hz line-broadening. The resulting frequency domain spectra were calibrated by setting the chemical shift of the

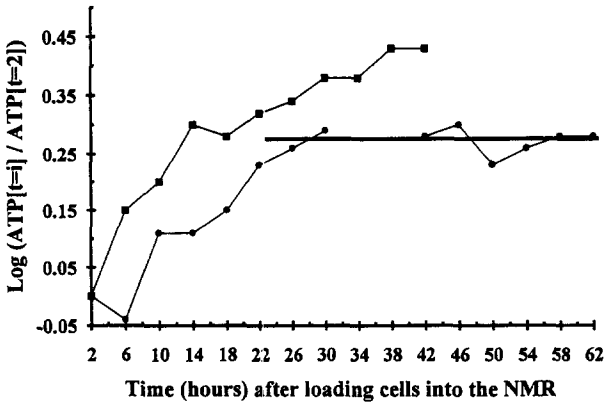


Fig. 1. Growth curves for 3T3 BALB/c cultures. The ordinate represents the logarithm of the ratio between two intensities of the β-ATP peak. One was taken at a given time, ATP($t = i$), and the other, ATP($t = 2$), was recorded immediately after loading the cells and served as a reference value. Normal proliferating cells display continuous growth (■), while cells treated with serum-deficient medium (●), enter the G_0/G_1-phase after 28 h. The horizontal line drawn inside the figure, represents a best-line fit (mean = 0.0001, $r = 0.992$) of all points within the 26–62 h time span corresponding to the presumed cell arrest period.

glycerophosphorylcholine peak to 3.02 ppm up-field from the phosphocreatine (PCr) peak.

To ensure normal gross rate for the perfused cell culture and to determine the time of complete arrest of cell division, we obtained sequential ^{31}P spectra for up to 48 h after loading the culture into the NMR magnet. The data are plotted as the logarithm of the β-ATP resonance intensity versus the time (Fig. 1).

^{23}Na spectra

Spectra were recorded with a spectral window of 4200 Hz using 4000 data points, 90° RF pulse (37 μs) and 200 transients spaced 0.1 s apart. The time domain data was zero-filled to 8192 data points and transformed with a 30 Hz line broadening function (Fig. 2). Respective peak areas were integrated and normalized using the software routine supplied with the instrument.

Sodium flux experiments

After adding of the SR (≈ 8 mM) to the circulating medium, we waited some 10–15 min to allow for complete separation of the intracellular Na^+ resonance from the extracellular Na^+ resonance (Fig. 2). Together with the SR, we added the flux's inhibitors at final concentrations of 0.0015 mg/ml for the bumetanide, of 0.25 mg/ml for the amiloride and of 0.7 mg/ml for the ouabain. When all the reagents were evenly spread over the culture, an initial set of 5–8 sequential ^{23}Na-NMR spectra was recorded. It was used in the determination of the baseline value of the serum-deficient intracellular Na^+ pool. An FCS bolus (10%, v/v) was added to the circulating medium; it was immediately followed by a fully automated acquisition of 50–80 successive ^{23}Na-NMR spectra (30 s each). The integrated value of the intracellular Na^+ peak, in each spectrum, was normalized with respect to its serum-deficient baseline value.

Since the SR may leak through the cellular membrane, we performed a long term experiment. The viability of the cell culture in the presence of the SR was monitored by acquiring both the ^{31}P-NMR and the ^{23}Na-NMR spectra at regular intervals throughout the experiment. Analysis has indicated that at the 5 h mark, only a minor SR leakage was observed. Complete leakage of the SR into the cells occurred only after an overnight perfusion (19 h). Therefore, we assumed that no SR leakage or related cellular damage would take place during the first 40–50 min of the ^{23}Na-NMR experiment.

2.3. Cell extracts for ^{31}P-NMR studies

Cell extracts were prepared immediately at the end of the ^{31}P-NMR measurement, by rapidly transferring the cell containing beads to a 50 ml of stirred ice-cold (v/v) perchloric acid ($HClO_4$) solution. The extracted cells were centrifuged in the cold and the acidic supernatant was neutralized with potassium hydroxide (KOH) solution. After the initial precipitation of the salt ($KClO_4$), the supernatant was lyophilized and re-dissolved in ≈ 1.5 ml of water. It was allowed to stand on ice for 1 h and the excess $KClO_4$ salt was filtered out. The paramagnetic impurities were removed from the clear supernatant by passing it through a short ion-exchange column (Chelex-100). The volume of the pooled eluates was re-adjusted to 3 ml in 20 mM EDTA and brought to pH 8–8.2. To prepare the sample for the NMR study, we repeatedly freeze-dried it with D_2O and re-suspended the residue in 3 ml of high-grade D_2O. The ^{31}P-NMR spectrum of each extract was recorded under the conditions described above, but with

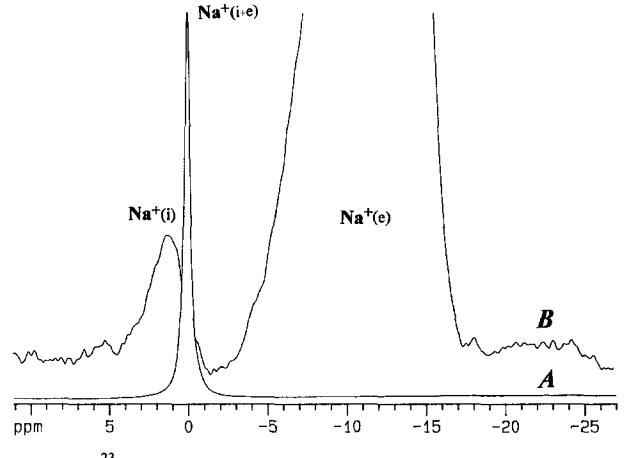


Fig. 2. The ^{23}Na-NMR spectra, taken at 106 MHz, recorded for the fully quiescent cell culture. The serum-deficient spectrum (A) was obtained just before adding the $Dy(ppp)_2^{7-}$ shift reagent to the medium. Separation of the intracellular Na^+ resonance from the bulk Na^+ resonances is obtained in the presence of the shift reagent (B). The vertical scale of the latter spectrum was increased to display the small, unshifted intracellular Na^+ peak.

longer recycle time (3 s). To allow chemical shift calibration and positive identification of the NMR resonances, small amount of solid UDP-glucose (70 μmol) was added to one of the extract samples.

2.4. Statistical analysis

All measurements were performed in triplicates. The ^{31}P-NMR data-based ordinate values used in the figures are given with a standard deviation of $\pm 10\%$ of the plotted points. The corresponding standard deviation in the case of the ^{23}Na-NMR data is $\pm 7\%$ of the plotted points. The data sets obtained for the serum-only case and for the three inhibitors' cases were analyzed by single factor ANOVA. We have demonstrated a significant statistical difference between data sets using both statistical correlation and regression analysis (see Section 4).

3. Results

3.1. Determination of the cellular arrest conditions within the NMR tube

Without published data, it was necessary to first establish the full arrest conditions for the perfused 3T3 fibroblasts culture while inside the NMR magnet. To this end, we performed ^{31}P-NMR studies using the following cell cultures: a proliferating culture, a quiescent culture, a proliferating culture treated with serum-deficient medium and a quiescent culture treated with serum-rich medium. In each case, the perfusion was maintained long enough to establish either full arrest or cell confluence conditions.

The ATP signals that appear in the ^{31}P spectra were used as markers indicating cell proliferation [21]. The method is based on the assumption that the cellular ATP pool in each cell does not change with time; therefore, an increase over time in the ATP signal intensity represents a net increase in the number of cells. Taken at regular intervals, the intensity of the β-ATP and γ-ATP peaks in the spectra were compared with that recorded for the serum-deficient case within the first 3 h following the start of cell perfusion. A plot of the signal intensity's logarithm versus the time, indicated that proliferating cells continued to divide inside the NMR tube for up to 48 h following the onset of perfusion (Fig. 1). Similar experiment established that healthy dividing cells became quiescent within 28–30 h from the initiation of the cell arrest (Fig. 1). As expected, by treating quiescent cells with serum-rich DMEM, an immediate exit from the G_0/G_1 phase was observed.

These results set up the basis of our experimental protocol for loading the cells into the NMR tube and defined the initial perfusion period needed prior to the ^{23}Na-NMR experiments (see Section 2).

3.2. Detachment of perfused quiescent cells in the NMR tube

Generally, normal proliferating cells firmly adhere to the beads and can successfully be perfused inside the NMR tube. In serum-deficient medium, cells entering the G_0/G_1 phase assume a rounder shape with dramatically reduced bead surface adhesion. To circumvent the problem, we reduced the serum concentration in two stages, from 10% to 5% and from 5% to 0.5%. Each stage was accomplished in 20–24 h, with the last one occurring in the NMR setup.

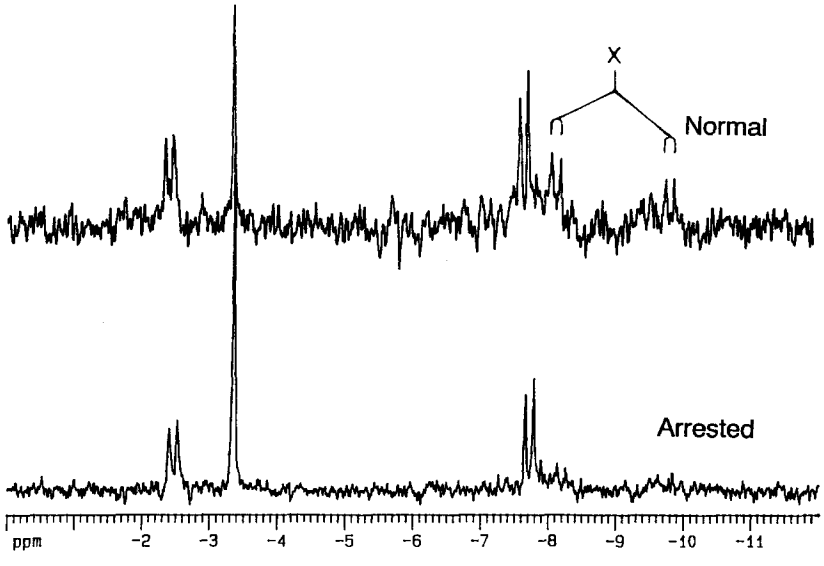


Fig. 3. The ^{31}P-NMR spectra, taken at 162 MHz, recorded using the perchloric extracts of proliferating cells (Normal) and of quiescent cells (Arrested). The X denotes the resonances ($P_\alpha = -8.17$ ppm and $P_\beta = -9.87$ ppm) that are tentatively assigned to UDP-*N*-acetylglucosamine. The same resonances almost disappear from the spectrum of the quiescent (Arrested) cells' spectrum.

3.3. ^{31}P-NMR studies of proliferating versus quiescent cells

In cells entering the G_0/G_1-phase, we observed a noticeable intensity decrease of the resonances corresponding to the sugar nucleotides in the cell (Fig. 3). To identify the compound, a perchloric acid extraction of the cell culture was performed. Assignment to UDP-glucose was ruled out by doping the sample with synthetic UDP-glucose. The position of the observed NMR resonances (P_α: −8.17 ppm and P_β: −9.87 ppm) relative to the doped UDP-glucose signal, tentatively identify the sugar nucleotide as UDP-*N*-acetylglucosamine [26]. With $\Delta_{\alpha\beta} = 1.70$ ppm, an assignment to UDP-glucuronate ($\Delta_{\alpha\beta} \approx 1.69$) was discarded because its resonances normally appear at the down-field side, rather than at the up-field side, of the UDP-glucose signals.

3.4. ^{23}Na-NMR studies of quiescent cells stimulated by serum

The suitability of each cell culture preparation for the study was ensured by recording its ^{31}P-NMR spectrum just before performing the ^{23}Na-NMR experiment. Five different experimental protocols with the perfused quiescent cells were performed using ^{23}Na-NMR. In the first protocol, cell division was induced by adding FCS to the circulating serum-deficient medium. This resulted in a transient increase of the intracellular Na^+ that crested at ≈ 140% some 6–8 min later. It was followed by slow decline back to baseline level within 20 min from the start (Fig. 4). In two other protocols, either amiloride or bumetanide was added together with the SR. Throughout the experiment, no significant change in the intracellular

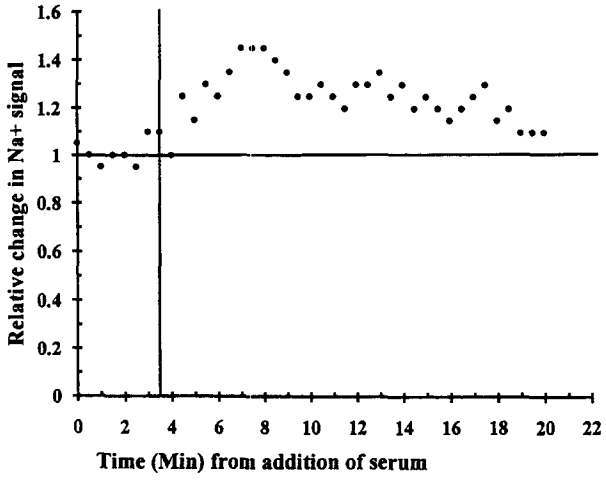


Fig. 4. A time-course plot of the (normalized) intracellular Na^+ in quiescent cells that exit the G_0/G_1-phase. The horizontal line represents the baseline of the intracellular Na^+ pool just before adding the FCS (10%) to the circulating medium. The vertical line denotes the approximate point, in time, when the serum-rich medium front actually reaches the perfused cells.

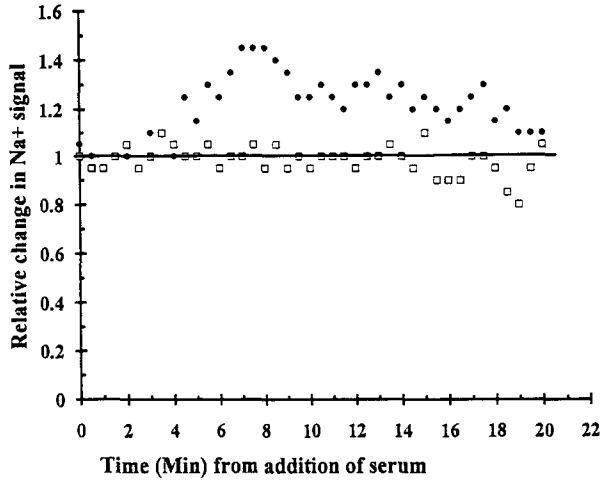


Fig. 5. A plot of the normalized intracellular Na^+ in quiescent cells that exit the G_0/G_1-phase in the presence (□) and absence (●) of amiloride (see Fig. 4).

Na^+ was observed when amiloride was added to the medium (Fig. 5). A small transient decline to < 80% was observed for the intracellular Na^+ when bumetanide was present in the medium (Fig. 6). In the fourth protocol, both flux inhibitors were added to the quiescent culture. Compared with the effects observed in the two previous cases, a non additive effect was observed when adding both drugs (Fig. 7 versus Figs. 5 and 6). In the last protocol, ouabain was present in the cell culture and the initial rise of the intracellular Na^+ was maintained throughout the experiment (Fig. 8).

One must notice that the true shape of the cellular Na^+ pool versus time plot (Fig. 4) is partially masked by two experimental factors. These are expressed as an apparent lag time in the onset of the Na^+ influx and as a reduced

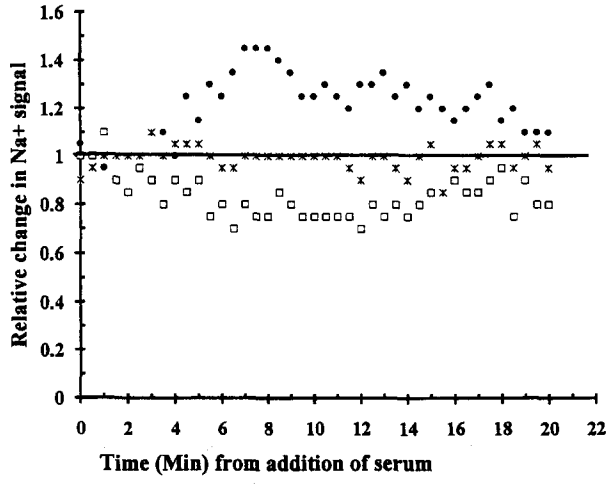


Fig. 6. A plot of the normalized intracellular Na^+ in quiescent cells that exit the G_0/G_1-phase in the presence (□) and absence (●) of bumetanide (see Fig. 4). A plot of the normalized Na^+ in quiescent cells continuously perfused with the serum-deficient medium in the presence of bumetanide (×) serves as secondary control (see text).

total increase in the intracellular Na^+ compared with the wet biochemistry results [14]. The apparent lag time is explained by the experimental design that dictates that it will take 3 min for the fresh medium front to flow through the extended tubing until it reaches the culture occupied space. The smaller magnitude of the increase, 140% versus 250%, reported previously by Panet et al. [14], is explained by the technical set up of the experiment as follows: the fresh medium enters the culture from the bottom and it takes up to 2 min for it to reach the top of the cell culture column. Thus, while the bottom cells are being stimulated by the oncoming serum front, the top cells still remain in quiescent state. Similarly, Na^+ efflux will first begin at the bottom cells spreading up toward the top of the culture column. The maximum influx point and the shape of the experimental curve (Fig. 4) do indicate that the Na^+ efflux must start within a short time following the cell stimulation (< 4 min). Since the NMR signal is being collected from all parts of the culture simultaneously, a net attenuation of the recorded NMR signal is observed during the increase or the decrease of the cellular Na^+ pool. Please note that the arrangement of the cell culture in a cylindrical shape is a requirement of the NMR equipment. At the same time, the NMR experiment provides a unique and reliable mean of monitoring variations in the intracellular sodium pool under in vivo conditions from the start till the end of the experiment.

4. Discussion

4.1. Quiescent cells have modified energy metabolism

We used ^{31}P-NMR to monitor the time-course of the cells entering the G_0/G_1 phase. Most of the observed spectral changes were easily accounted for either by an

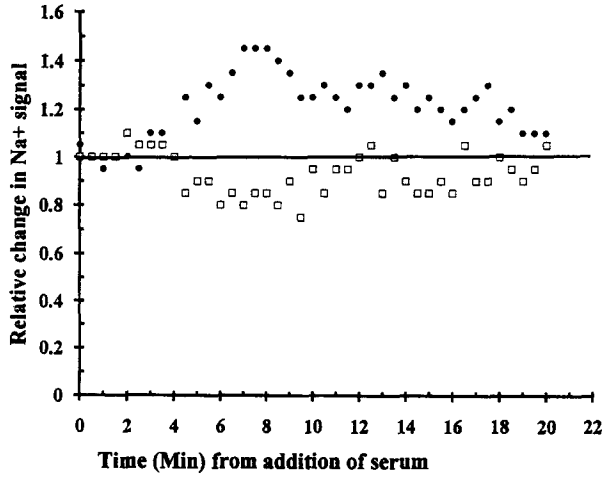


Fig. 7. A plot of the normalized intracellular Na^+ in quiescent cells that exit the G_0/G_1-phase in the presence (□) and absence (●) of amiloride and bumetanide (see Fig. 4).

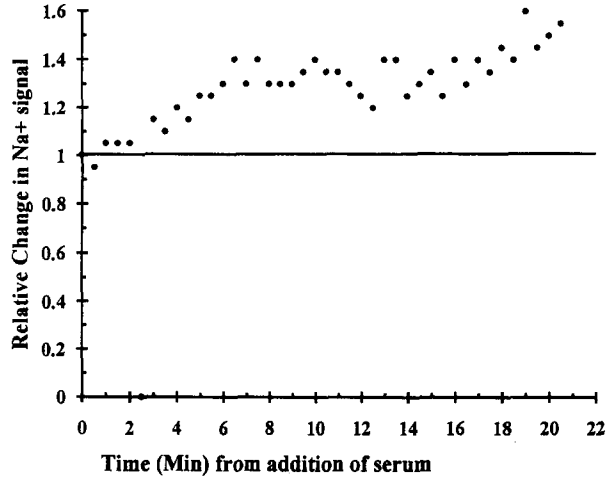


Fig. 8. As in Fig. 4, but in the presence of ouabain (●).

increase in total cell count, or as a result of a critical depletion of nutrients [26]. Unusually, we have observed a marked decrease in the intensity of the NMR signals corresponding to sugar nucleotides (mainly UDP-hexoses). Most of the intensity loss occurred just before the culture became fully quiescent. To provide better resolution within the area of interest in the ^{31}P-NMR spectra, we have performed a perchloric extraction on the proliferating and on the fully quiescent cell cultures (Fig. 3). The dependence of the levels of UDP-hexoses on the medium formulation and on the serum type in cultured cells was recently demonstrated [26]. Reducing the amount of the supplied FCS in the medium, from 10% to 2.5% serum, lead to an increase in the UDP-hexose levels in the cells [26]. In the present case, the opposite trend was observed when the culture was perfused by the serum-deficient DMEM (0.5% FCS). The apparent reduction in the UDP-hexose levels could be a result of an eventual shortage of an essential metabolite from the medium formulation. However, we did not detect similar reduction in healthy proliferating cell cultures. Alternatively, the reduction may mean that the steady-state concentration of certain UDP-hexoses is lower in quiescent cells compared with normal cells.

4.2. The increase in Na^+ influx is induced by serum stimulation of quiescent cells

Elevation of the intracellular Na^+ after adding FCS was sustained for only a short period of time and was followed by a steady decline to base level (Fig. 4). As previously observed in these cells, the initial increase in the Na^+ pool was an expected result of the mitogenic signal [14]. The observed decline in the cellular Na^+ could only be intuitively expected. The initial influx of Na^+ into the cell is expected to stimulate the Na^+/K^+-ATPase pump leading to a subsequent efflux of Na^+ from the cell.

By ignoring the lag time required for the serum-rich

medium in the reservoir to reach the cells, we observed that the intracellular Na^+ rises to a peak value in 4 min. It is then followed by a decline to the baseline within the next 10 min. Similar response curve was previously observed in wet experiments using the ^{86}Rb tracer [8]. Unlike the present case, in the only paper that reported direct measurement of the intracellular Na^+ by atomic absorption, the steady state Na^+ pool was shown to remain high for as long as 30 min after the addition of the serum [14].

4.3. Ouabain blocks subsequent Na^+ efflux from quiescent cells stimulated by serum

Our initial hypothesis, that stimulation of the Na^+/K^+-ATPase pump is responsible for the Na^+ efflux, was confirmed by adding ouabain that inhibits the Na^+ efflux. In the presence of ouabain, the transient increase in the intracellular Na^+ was maintained (Fig. 8), clearly indicating that the efflux in uninhibited cells is due to the Na^+/K^+ ATPase pump.

4.4. Flux inhibitors block Na^+ influx into quiescent cells stimulated by serum

Addition of either amiloride or bumetanide

The complete blocking of the Na^+ influx by either bumetanide (Fig. 5) or amiloride (Fig. 6) indicates that inhibition of one ion transporter also effects the Na^+ influx through the other transporter. Our observations support the notion that following the cellular exit from the G_0/G_1-phase, both the Na^+/H^+ antiport and the $Na^+/K^+/Cl^-$ cotransport are actively contributing to the early increase in the intracellular Na^+.

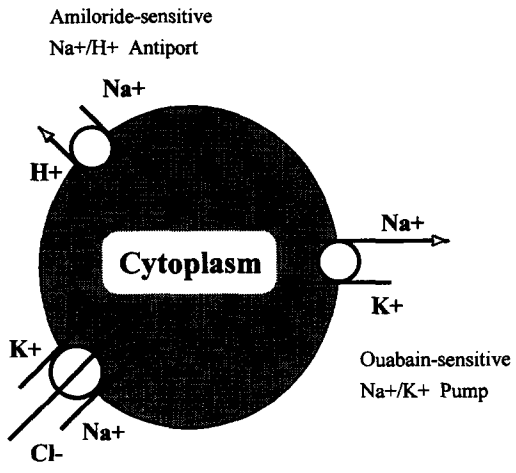
The reason for the transient efflux of intracellular Na^+, in the presence of bumetanide, is not clear to us (Fig. 6). To ensure that it is a direct response to the mitogenic signal, the same experiment was repeated but without adding the serum to the culture. No change in the Na^+ baseline level was detected (Fig. 6) and a best line fit of the data yielded a slope of -0.0006 and an intercept of 0.997.

Addition of both amiloride and bumetanide

The above results lead us to expect that the addition of both amiloride and bumetanide to the culture would also result in a complete inhibition of the sodium influx. A non-additive effect of the two inhibitors was indicated from the data since it could fit an averaging of the two individual effects (Fig. 7).

Analysis of the flux inhibitors' data

To establish the significance of the above results, we have performed statistical analysis of the experimental data. A single-factor ANOVA analysis of the above four data sets yielded $P \leq 0.0001$ at 95% confidence level. Student's t-test analysis of the serum-only data and the



Scheme 1.

'nearest in magnitude' amiloride data yielded a $P \leq 0.0001$. With correlation coefficients of 0.35–0.05, the statistical independence of the data was confirmed also from the regression analysis of data. In the presence of amiloride, the cellular Na^+ remains near baseline (mean = 0.983, var. = 0.004). Addition of bumetanide to the medium resulted in a net decrease of the intracellular Na^+ (mean = 0.832, var. = 0.008). When both flux inhibitors are present in solution, the intracellular Na^+ pool represents approximately an averaged value between the two previous cases (mean = 0.930, var. = 0.015).

5. Conclusions

The NMR experiment allowed us to record the steady-state concentration of the intracellular Na^+ that is represented by an averaging of its influx and efflux through the cell transporters. The present work represents a direct assessment of the early increase in intracellular Na^+ produced by addition of serum to quiescent cells. The results also support the notion that the effects of these transporters are not necessarily independent of each other.

Each of the two sodium transport inhibitors prevented the rise of the intracellular Na^+ that is normally produced by FCS stimulation of quiescent cells. However, their mechanisms of action seem to differ. The amiloride inhibited the rise of the intracellular Na^+, while the bumetanide caused a small transient decrease of the Na^+ pool. These differences point to the possible existence of complex coupling between the sodium transporters and the subsequent activation of the second messenger pathways by growth-factors.

Previous works [15–17] have shown that prevention of the early increase of Na^+, by either amiloride and bumetanide alone or in combination, resulted in DNA

synthesis inhibition 18 h later, that was reversible upon elimination of the inhibitors. The data presented in this study lead to the following conclusion: a transient increase in the intracellular Na^+ is produced by serum stimulation through activation of the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport and of the amiloride-sensitive Na^+/H^+ antiport (see Scheme 1). Under normal conditions, this increase does not persist more than a few minutes because of the triggering of the Na^+/K^+-ATPase responsible for pumping out the intracellular Na^+ (see Scheme 1). Thus, a correlation exists between the triggering of a transient increase in sodium content by addition of serum and the inhibition of mitogenesis when this increase is inhibited by the blocking of two different ion transport pathways. Of course, we cannot rule out the possibility that the inhibitors of sodium transports have additional toxic effects able to block mitogenesis in a similar reversible fashion. Inhibition of protein kinases by amiloride has been reported in different cell types and under different experimental conditions [27,28]. However, no toxic effect of that sort has been reported for bumetanide that seems to be more specific for the $Na^+/K^+/Cl^-$ cotransport.

In view of the clear correlation mentioned above, the possibility for a transient change in Na^+ content to be part of the early mitogenic signal inducing a cascade of reactions leading to DNA synthesis and cell division hours later cannot be ruled out either.

It could be argued that the existence of cell mutants that are defective for these ion transport pathways [19] indicates that the latter are not necessary for cell division. However, the same is true for mutants that are defective in other second messenger systems [29]. These observations only show that the second messenger systems have a certain degree of redundancy that may be used differently in different cell types. The large variety of possible cooperative effects between them explains how the diversity and specificity in receptor activation are obtained despite the small number and low specificity of second messengers. Therefore, the activation of an alternative system is likely to compensate for lack of a transporter in a defective cell [30,31]. Under the conditions of this work the $Na^+/K^+/Cl^-$ cotransport and the Na^+/H^+ antiport seem to work in series, since the effects of their inhibitors are not additive: one is enough to totally block the stimulation of sodium entry. However, it is possible that a coupling of a different sort, in parallel, takes place in different cell types [31] so that the inhibition or deficiency of one would be compensated for by the other. A further way to test this hypothesis is to look for a transient change in Na^+ content similar to the one reported here, in different cell types including defective mutants.

In addition, a plausible hypothesis must be worked out and tested regarding a mechanism whereby a transient change in sodium content could produce a new physiological cellular state. Sodium ions share with calcium ions the property of very low normal cytoplasmic concentration. Both ions are very sensitive sensors and important relative changes in their concentrations are easily produced by small quantities of ions entering or leaving the cytoplasm. However, contrary to what is the case for calcium ions, a possible involvement of sodium in the mitogenic signal is not necessarily restricted to the activation or inactivation of one or a few enzymes. Sodium may also be effective in a non-specific way by changing the overall ionic strength of the intracellular medium and/or its osmotic pressure and thereby the cell volume. The role of bumetanide sensitive cotransport on volume regulation is well documented [10,13,19,32]. However, the transient nature of the sodium change reported here restricts the effect of such a mechanism to a few minutes after addition of serum. As a possible mechanism, one may consider a rapid change of state from an attractor to another in the dynamics of a network of metabolic reactions. Such a switch from a stable state, where a given pattern of reactions is active and others inactive, to a different stable state with a different pattern of activities is produced when the concentration of some metabolites reaches a threshold value. Rapid changes in concentration do not have to be produced by specific changes in some enzymatic activities, but merely be produced by transient changes in cellular volume. The specificity of the observed effects does not result from the specificity of the signal but from that of the dynamics of the network of reactions. Such mechanisms have been increasingly documented [33]. Their low specificity is what insures their redundancy, i.e., the possibility of alternative ways to produce the same final state.

Further work must be done to study more precisely the implications of this hypothesis. Namely, the effects of different individual growth factors must be studied. Changes in cellular sodium content similar to the one reported here must be looked for in different cell types. Monitoring transient changes in cellular volume under different cellular growth conditions would allow to correlate them with observed changes in sodium content.

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