Increase in Intracellular Na\(^+\): Transmembrane Signal for Rejoining of DNA Strand Breaks in Proliferating Lymphocytes

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ABSTRACT

Two early events in the mitogen-induced entry of murine splenocytes into proliferation are (a) a rapid rise in influx of Na\(^+\), causing its total internal concentration to increase by 42% within 2 h of culture with concanavalin A (Con A), and (b) rejoining of some 3000 DNA strand breaks per diploid genome within the same period. Con A did not induce rejoining in low Na\(^+\) (<9 mM) medium, but the process began directly when Na\(^+\) was added, at its usual concentration, to the growth medium. Incubation of cells with ouabain, an inhibitor of the Na\(^+\) K\(^-\)-ATPase, or monensin, a Na\(^+\) ionophore, caused an increase in the internal Na\(^+\) concentration in normal, but not in low, Na\(^+\) medium. In the former (but not in the latter) medium, both ouabain and monensin caused rejoining of the DNA strand breaks to occur in resting lymphocytes, i.e., in the absence of mitogen. Stimulation of splenocytes with Con A also resulted in a rapid but transient increase in the level of intracellular free Ca\(^{2+}\). This effect was also observed in the absence of extracellular Na\(^+\); however, deprivation of extracellular Ca\(^{2+}\) completely abolished this effect. Moreover, the intracellular free Ca\(^{2+}\) level was significantly higher in cells suspended in Na\(^+\)-free buffer or medium. Since the Con A-induced rejoining of DNA strand breaks occurred in the absence of extracellular Ca\(^{2+}\) and removal of extracellular Na\(^+\) had no inhibitory effect on the Con A-induced increase in the level of intracellular free Ca\(^{2+}\), the Con A-stimulated repair could not have been mediated by the initial increase in Ca\(^{2+}\) influx. The early mitogen-induced increase in the internal Na\(^+\) concentration is a necessary and sufficient signal for the rejoining of breaks, an event that must occur before the proliferating lymphocytes can replicate their DNA.

INTRODUCTION

Central problems in the cell biology of proliferation are the elucidation of the mitogenic signal that triggers a resting cell to enter the proliferative cycle, as well as the identification of the cascade of biochemical events that link this signal to the replication of the genome that may occur many hours later. In the case of the lymphocyte, the existence of two mitogenic signals is usually presumed: the first is the binding of mitogen or antigen to receptors at the cell surface; and the second is the binding of the lymphokine IL 2 to its newly synthesized membrane receptors (1). However, transduction of the proliferative signal is in fact much more complex than the simplistic two-signal model suggests. In addition to binding of mitogen and IL 2, the transmembrane proliferative signals include, among many other things, the rapid movement of Na\(^+\) into the lymphocyte with a consequent increase in its total Na\(^+\) (2-5). The increased Na\(^+\) is responsible for the early increase in active K\(^+\) influx during lymphocyte proliferation noted many years ago (2, 4, 5); these ionic events are essential to entry into the proliferative cycle. The rise in [Na\(^+\)], causes an immediate increase in the activity of the Na\(^+\)-K\(^-\)-ATPase of the cell membrane, before a significant rise in the number of Na\(^+\)-K\(^-\) pump sites occurs (6).

An increase in f(Ca\(^{2+}\)) occurs soon after mitogenic stimulation of lymphocytes (7, 8), and this has also been implicated in the regulation of several cellular processes which are necessary for proliferation (9). Recent data (10, 11) are consistent with the hypothesis that the increased Ca\(^{2+}\) observed in stimulated lymphocytes could be the trigger for the activation of the protooncogenes c-myc and c-fos.

Another essential event that precedes blast formation and replication of the genome in human (12), murine (13), and porcine (14) lymphocytes is the rejoining of DNA strand breaks present in the resting cells. In the case of mouse lymphocytes, some 3000 DNA strand breaks per cell are rejoined within 2 h of the addition of Con A (13, 14). The hypotheses that these breaks were either artifacts of the methods of extraction and assay or were akali-labile bonds were excluded by experimental test (15). In all of the species so far examined, rejoining of the breaks involves the activity of poly(ADP-ribose) synthetase (EC 2,4,99). Inhibitors of this enzyme prevent the DNA strand breaks from rejoining and the cells from undergoing blastogenesis and entry into S phase; all of these effects are freely reversible (15). Rejoining of the strand breaks is not required for blast transformation but is essential to replication of the DNA (15). However, nothing is known as to the actual mechanism by which Con A induces the rejoining of strand breaks.

Studies demonstrating the introduction of single strand breaks during the differentiation of primary chick myoblasts into myotubes (16) and of Friend erythroleukemia cells (17) raise the possibility of a regulatory role for DNA strand breaks in differentiation. It is interesting that 12-O-tetradecanoylphorbol-13-acetate, a cocarcinogen, is known to induce DNA strand breaks in human leukocytes (18).

We now demonstrate that it is the increase in [Na\(^+\)], caused by proliferative activation of the lymphocyte, that is the transmembrane signal for the rejoining of these DNA strand breaks; indeed, the breaks undergo rejoining in the absence of mitogen when a rise in [Na\(^+\)], is provoked by a sodium ionophore or by inhibiting the Na\(^+\)-K\(^-\)-ATPase (EC 3,6,1,3) of the cell membrane.

MATERIALS AND METHODS

Reagents. [methyl-\(^{3}H\)]Thymidine was purchased from New England Nuclear. Ouabain, monensin, Triton X-100, Con A, diethylene triaminepentacetetic acid, EGTA, and ethidium bromide were obtained from Sigma. MBA was purchased from Aldrich Chemical Company, Inc. Tissue culture media and reagents were obtained from Gibco and trypan blue from MCB Reagents, Ltd. Quin 2 and Quin 2/AM were purchased from Calbiochem and Chelex 100 from BIORAD.

Preparation and Culture of Mouse Splenocytes. Spleens of male BALB/c mice, 8 to 12 wk old, were used for the preparation of cells; details have been reported elsewhere (19). Experiments were carried out later the same day, after allowing the cells to sit at 37°C, in 5%...
controls. Any value below zero indicates an increase in the number of breaks.

Bars, SD.

were calculated as the change in the number of breaks compared to resting

and incubated at 37 °C for 2 h in low sodium medium. They were then centrifuged

or absence of Na+. Freshly harvested lymphocytes were stimulated with Con A

changes were measured.

Compounds but does not give the absolute number of strand breaks

stimulated with Con A for the required time, spun, and resuspended in normal or Na+-free buffer, and then the Ca2+-related fluorescence

(5). A value of 0.15 ´il per 10^9 cells as the volume of intracellular water

genome (22)]. This method only permits the measurement of change in

method which does not involve the use of high pH to denature DNA

tion curve obtained from cells treated with various doses of Î³ radiation

completely inhibited by MBA, an inhibitor of poly(ADP-ribose)

subsequent entry into S phase (see Table 1); indeed, amiloride itself provoked the appearance of a significant number of breaks (Table 1). Amiloride has other side effects (see below) so that these data must be interpreted with caution. Addition of 10 mM

NH4Cl for 2 h to resting mouse splenocytes had no effect on

ing of DNA strand breaks had occurred at 2 h after the addition

pig lymphocytes (5). As reported earlier (13), maximum rejoin

nocytes, since this effect is not seen in cells stimulated in low Na+ medium. This had already been shown in phytohemagglu-

was adjusted to 11.0. Under these conditions, ethidium bromide

selectively binds to double-stranded DNA. Based on the percentage of
double-stranded DNA remaining after alkali treatment, one can estimate
the change in the number of DNA strand breaks, using a calibration curve

from obtained from cells treated with various doses of γ radiation

[500 rads of γ radiation produce 6000 single-strand breaks per diploid genome (22)]. This method only permits the measurement of change in

the number of strand breaks after treatment of cells with various compounds but does not give the absolute number of strand breaks

present. Similar results were obtained with the nucleoid sedimentation

method which does not involve the use of high pH to denature DNA

to sedimentation (12, 15).

Measurement of [Na+]i. Details of the method are described elsewhere (5). A value of 0.15 ´il per 10^9 cells as the volume of intracellular water

of mouse splenocytes was used for the preparation of the molar concentration of intracellular [Na+]. No change in the cell volume after

Con A treatment for 2 h was observed.

Measurement of [Ca2+]i. This was exactly according to the procedure

of Tsien et al. (7, 8). Na+-free buffer contained an equimolar amount

of choline chloride in place of NaCl, and Na2HPO4 was replaced by

K2HPO4 maintaining the overall [K+] at 5 mM. Ca2+-free buffer was

prepared by omitting CaCl2. Glass distilled water treated with Chelex

100 was used for the preparation of all buffers.

In experiments involving the measurement of [Ca2+]i, at ½ and 2 h

after stimulation of the splenocytes with Con A, Quin 2-loaded cells

were resuspended in either normal medium or low Na+ medium,
stimulated with Con A for the required time, spun, and resuspended in normal or Na+-free buffer, and then the Ca2+-related fluorescence

changes were measured.

RESULTS

Fig. 1 shows the results of an experiment in which mouse splenocytes were stimulated with mitogenic concentrations of

Con A in low (≤9 mM) Na+-medium, and the number of DNA strand breaks rejoined was measured as described. It is clear that, under these conditions, Con A could neither induce the usual rejoicing of DNA strand breaks (Fig. 1) nor could it effect an increase in [Na+], (Table 1). The block was freely reversible

upon replacing the cells in normal medium containing Con A at mitogenic concentration; some 4000 DNA strand breaks were repaired within 1 h (Fig. 1). These data suggested that the trigger for DNA strand break rejoining might be the mitogen-induced increase in [Na+]. This hypothesis was tested by a series of experiments involving study of the action of agents previously shown (5) to cause a rise in the [Na+], of resting lymphocytes; this was the case of ouabain, the inhibitor of Na+-K+ ATPase, and of the Na+ ionophore monensin.

Table 1 shows that ouabain, present in its usual inhibitory concentration in normal medium for 2 h, caused the [Na+], of resting cells to increase by more than 2-fold; monensin caused a 4-fold increase. Treatment with either inhibitor resulted in the disappearance of a large number of DNA strand breaks in resting lymphocytes, a number not significantly different from those that were rejoined in cells induced to proliferate by Con A (Table 1). However, neither ouabain nor monensin caused the lymphocytes to enter S phase, as shown by Table 1. Amiloride, an agent that blocks Na+/H+ antiport (23), prevented the rise in [Na+], caused by Con A in normal medium and also blocked the Con A-induced DNA strand break rejoicing and subsequent entry into S phase (see Table 1); indeed, amiloride itself provoked the appearance of a significant number of breaks (Table 1). Amiloride has other side effects (see below) so that these data must be interpreted with caution. Addition of 10 mM

NH4Cl for 2 h to resting mouse splenocytes had no effect on

the rejoining of DNA strand breaks (data not shown).

Table 2 shows that, like the Con A-induced disappearance of strand breaks, that induced by ouabain or monensin was also completely inhibited by MBA, an inhibitor of poly(ADP-ribose) synthetase, suggesting that the mechanism of rejoicing involves this enzyme system in all cases.

Since Con A is known to cause a rise in f[Ca2+], (7, 8), it seemed possible that the rejoining of DNA strand breaks was mediated by a rise in f[Ca2+]. Table 3 shows that the Con A-induced increase in f[Ca2+] in low Na+ buffer as well as in normal; a small increase in f[Ca2+] was induced in resting cells in normal Na+ buffer by monensin but not by ouabain. The relatively high levels of f[Ca2+] found in lymphocytes incubated in low Na+ buffer were striking and are consistent with a functioning Na+/Ca2+ exchange system in these cells. The rejoining of strand breaks induced by Con A in Ca2+-free medium was not significantly different from that in Ca2+ -containing medium. In three experiments, 2050 ± 174 DNA (SD) strand breaks were rejoined in cells incubated with a mitogenic concentration of Con A in normal medium and 1920 ± 66 in cells incubated in medium containing 2 mM EGTA, sufficient to remove all the Ca2+ therein. In Ca2+-free buffer, the usual Con A-induced increase in f[Ca2+] did not occur, suggesting that this phenomenon is caused by uptake of Ca2+ from the medium. Data of this kind support the conclusion that f[Ca2+] is not involved in the induction of rejoicing of the DNA strand breaks.

DISCUSSION

It is clear that an increase in Na+ influx is responsible for the increase in [Na+], observed in Con A-stimulated mouse splenocytes, since this effect is not seen in cells stimulated in low Na+-medium. This had already been shown in phytohemagglutinin-stimulated human lymphocytes (2) and Con A-stimulated pig lymphocytes (5). As reported earlier (13), maximum rejoining of DNA strand breaks had occurred at 2 h after the addition of Con A to mouse splenocytes. However, in the case of cells
INTRACELLULAR Na* AND REJOINING OF DNA STRAND BREAKS

Table 1 Effect of Con A, ouabain, monensin, and amiloride on [Na*], DNA strand break repair, and [3H]thymidine incorporation in mouse splenocytes cultured in normal and low Na* medium

Con A (2.0 μg/ml), ouabain (0.5 mM), monensin (20 μM), and amiloride (0.5 mM) were used in these experiments. [Na*], and DNA strand breaks were measured at 2 h after Con A addition. In the case of the number of strand breaks rejoined, any value below zero indicated an increase in the number of breaks as compared to resting controls. [3H]Thymidine incorporation was measured at 48 h after Con A addition. Statistical significance was determined by the paired t test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Na*] (mM)</th>
<th>% of change in [Na*] compared to resting</th>
<th>No. of DNA strand breaks repaired</th>
<th>[3H]Thymidine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting</td>
<td>16.7 ± 4.1*</td>
<td>4.19 ± 7.4 (14)</td>
<td>3,160 ± 260 (14)</td>
<td>2,813 ± 200</td>
</tr>
<tr>
<td>Con A</td>
<td>24.1 ± 7.5 (14)</td>
<td>122.3 ± 14.6 (6)*</td>
<td>4,290 ± 1,940 (6)</td>
<td>237,000 ± 370</td>
</tr>
<tr>
<td>Ouabain</td>
<td>42.8 ± 14.9 (6)</td>
<td>405.9 ± 61.3 (4)</td>
<td>4,270 ± 1,260 (4)</td>
<td>1,800 ± 70</td>
</tr>
<tr>
<td>Monensin</td>
<td>85.2 ± 26.3 (4)</td>
<td>1.8 ± 11.0 (2)</td>
<td>−1,160 ± 400 (2)</td>
<td>610 ± 210</td>
</tr>
<tr>
<td>Amiloride</td>
<td>14.0 ± 3.5 (2)</td>
<td>4.0 ± 33.4 (2)</td>
<td>−1,090 ± 350 (2)</td>
<td>330 ± 20</td>
</tr>
<tr>
<td>Monensin + Con A</td>
<td>14.4 ± 3.0 (2)</td>
<td>42.3 ± 57.4 (4)</td>
<td>−1,200 ± 370 (4)</td>
<td>200 ± 50</td>
</tr>
<tr>
<td>Low Na* medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting</td>
<td>4.8 ± 4.5 (6)</td>
<td>0.3 ± 9.6 (6)</td>
<td>704 ± 900 (6)</td>
<td>500 ± 20</td>
</tr>
<tr>
<td>Con A</td>
<td>4.7 ± 4.3 (6)</td>
<td>4.4 ± 25.1 (3)</td>
<td>−960 ± 580 (3)</td>
<td>3,350 ± 170</td>
</tr>
<tr>
<td>Ouabain</td>
<td>6.7 ± 4.3 (3)</td>
<td>6.7 ± 4.5 (6)</td>
<td>−140 ± 400 (6)</td>
<td>200 ± 50</td>
</tr>
<tr>
<td>Monensin</td>
<td>5.9 ± 7.2 (4)</td>
<td>42.3 ± 57.4 (4)</td>
<td>−1,200 ± 370 (4)</td>
<td>200 ± 50</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Numbers in parentheses, sample size.
* Significantly higher when compared to corresponding resting cell value at P < 0.01.
* Not significantly different from corresponding resting cell values.

Table 2 Ouabain- or monensin-induced DNA strand break repair in the presence or absence of MBA in mouse splenocytes

Con A (2 μg/ml), MBA (5 mM), ouabain (0.5 mM), and monensin (20 μM) were used in the experiments. Cells were treated and incubated at 37°C and 5% CO2 for 90 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA strand break assay (% of double-stranded DNA after 90 min with Con A)*</th>
<th>Breaks repaired (change in no. of breaks compared to controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>40.8 ± 3.2*</td>
<td>−3.50</td>
</tr>
<tr>
<td>Con A</td>
<td>51.8 ± 3.3*</td>
<td>−640*</td>
</tr>
<tr>
<td>Con A + MBA</td>
<td>38.3 ± 2.6</td>
<td>2944</td>
</tr>
<tr>
<td>Ouabain</td>
<td>50.0 ± 0.6*</td>
<td>192</td>
</tr>
<tr>
<td>Ouabain + MBA</td>
<td>41.4 ± 2.6</td>
<td>2528</td>
</tr>
<tr>
<td>Monensin</td>
<td>48.7 ± 1.5*</td>
<td>−1408*</td>
</tr>
<tr>
<td>Monensin + MBA</td>
<td>36.4 ± 5.7</td>
<td>122</td>
</tr>
</tbody>
</table>

* Percentage of double-stranded DNA determined after the alkali treatment and not representative of the structure of DNA in situ.
* Mean ± SD, drawn from at least 3 different experiments.
* Significantly higher value compared to corresponding resting cell value at P < 0.05.
* Negative numbers indicate an increase in DNA strand breaks compared to controls.

previously exposed to low Na*-medium and restimulated with Con A in normal Na*-medium, a much more rapid rate of rejoining of DNA strand breaks was observed (Fig. 1). This could be due to a prior occurrence of Na+-independent events that contribute to the process of rejoining of the endogenous strand breaks.

That there is a causal relation between the rise in [Na*] and the rejoining of the endogenous DNA strand breaks is shown by several different types of data. (a) Con A induces an increase in [Na*], and subsequently the rejoining of DNA strand breaks in mouse splenocytes. (b) Monensin and ouabain, two structurally and functionally unrelated compounds, both cause an increase in Na* by different mechanisms and also effect the rejoining of DNA strand breaks in the absence of mitogen. (c) Inhibition of the Con A-induced increase in [Na*] by treatment with amiloride or by suspending the cells in low Na*-medium prevents the rejoining of DNA strand breaks.

Treatment with amiloride caused an increase in the number of DNA strand breaks, as seen from Table 1, thus adding yet another to the already imposing list of nonspecific effects of this compound (24–26). Although monensin and ouabain were as efficient as Con A in effecting the rejoining of DNA strand breaks, they also induced DNA strand breaks in cells suspended in low Na*-medium (Table 1). Normal [Na*] may thus have a protective effect on DNA, and lowering of [Na*] may result in enhancing the effect of various DNA-damaging agents. The fact that 10 mM NH4Cl had no effect on the number of DNA strand breaks in resting cells suggests that intracellular pH is not involved in the rejoining of DNA strand breaks (data not shown).

We confirm the finding (7, 8) that Con A is able to induce a rapid but transient increase in [Ca2*], in mouse splenocytes. Removal of extracellular Na* and/or addition of monensin and ouabain had no inhibitory effect on the Con A-induced increase in [Ca2*]. We also have presented evidence (Table 3) for the existence, in the plasma membrane of mouse splenocytes, of a
functional Na*/Ca** antiport that contributes to the efflux of intracellular Ca**, under physiological conditions. The presence of a Na*/Ca** antiport in the membrane vesicles of rabbit lymphocytes has been recently reported (27). Using Quin 2 AM-loaded cells, a rapid increase in fCa** was seen by 5 min after mitogen, but no further change was noted at 30 min or 2 h. The facts that (a) removal of extracellular Na* inhibited Con A-induced rejoining of DNA breaks but had no inhibitory effect on the Con A-induced increase in fCa**, and (b) the level of fCa**, in cells suspended in Na*-free buffer (in which no rejoining occurred) was close to that observed in cells suspended in normal Na* buffer rule out the possibility that increased fCa** was causally involved in the rejoining of DNA strand breaks observed in mitogen-stimulated lymphocytes.

That monovalent cations might serve as signals for proliferation has been frequently postulated (28–34). Several reports implicate a rise in [Na*], as causing changes at the level of the nuclear DNA. For example, in chick embryo fibroblasts, an increase in the number of amino acid transport (L-proline) sites occurred after a 4-h exposure of cells to high extracellular Na* (35); this increase was blocked by inhibitors of transcription. In the same cell system, a brief exposure to high extracellular Na* induced the appearance of DNase I hypersensitive sites that could be propagated to daughter cells in the absence of the original inducer (36); it was suggested that a transient increase in intracellular Na* might be the mediator of this phenomenon. Thus it appears that increased [Na*], might play an important role in activation of a number of nuclear events. In any case, all of the present data are consistent with the hypothesis that the mitogen-induced Na* influx, resulting in increased [Na*], is a sufficient signal for the rejoining of the strand breaks that must occur if the DNA is to replicate (15). We are at present determining whether the high [Na*], is a sufficient stimulus for the induction of other early nuclear events in the activation of proliferation.

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REFERENCES

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