Glucocorticoids Regulate Plasma Membrane Potential During Rat Thymocyte Apoptosis in Vivo and in Vitro

CYNTHIA L. MANN AND JOHN A. CIDLOWSKI

Molecular Endocrinology Group, Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, National Institutes of Health, Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

ABSTRACT

Glucocorticoids induce a series of profound biochemical changes in thymocytes that initiate apoptosis; however, the pathways beyond receptor transactivation that lead to this form of cell death are not fully understood. In this study, we report a novel site of action for glucocorticoids at the site of the plasma membrane. Specifically, we find that glucocorticoids induce the loss of plasma membrane potential both in vivo and in vitro. The glucocorticoid-induced loss of plasma membrane potential in cultured primary isolated thymocytes was both dose and time dependent. Other steroid hormones, including progesterone, estrogen, and testosterone, fail to alter the depolarization state of the thymocyte plasma membrane. Interestingly, other nonsteroid stimuli that also activate apoptosis in thymocytes also lead to cellular depolarization. In contrast, HeLa cells, which contain functional glucocorticoid receptors but do not die in response to hormone, do not alter their plasma membrane potential in response to glucocorticoids, indicating a strong association between depolarization and apoptosis. Furthermore, the ability of glucocorticoids to depolarize the plasma membrane of thymocytes required the interaction of glucocorticoids with their cognate receptor, because RU486 failed to depolarize thymocytes and antagonized the effect of glucocorticoids. Finally, experiments using inhibitors of transcription and translation indicated that the loss of plasma membrane potential in thymocytes following glucocorticoid treatment required de novo gene expression.

The results of these studies establish that the loss of plasma membrane potential is an important feature of glucocorticoid-induced apoptosis of thymocytes. (Endocrinology 142: 421–429, 2001)

HOMEOSTASIS of T cell number in the thymus is controlled by the interplay of proapoptotic and antiapoptotic factors that regulate the thymocyte population. The elimination of unwanted thymocytes by proapoptotic signals, including endocrine signals such as glucocorticoids, is crucial to maintaining the integrity of the immune system. Glucocorticoids induce a cell death program in primary isolated thymocytes that is biochemically and morphologically characterized by chromatin condensation, DNA fragmentation, caspase activation, and the loss of mitochondrial membrane potential (1–4). These cellular alterations are conserved features of apoptosis that are characteristic of apoptosis in many lymphoid and other cell types (5, 6).

In addition to these cytoplasmic and nuclear components, the plasma membrane of the cell undergoes profound changes during apoptosis. Early observations of apoptotic death described the cell membrane as blebbing and pinching off late in apoptosis to form inclusions termed apoptotic bodies (7, 8). The reorientation of phosphatidyl serine residues to the exterior of the plasma membrane aids in the recognition of the apoptotic cell by macrophages (9). In addition, glucocorticoids have been shown to decrease transmembrane transport of amino acids, glucose, and nucleosides (10). Previous studies also showed that apoptotic cells shrink (11) and suggested that this is due to the loss of fluid and ions from the cell (12). More recent studies have demonstrated that apoptotic cell shrinkage occurs as a result of ionic efflux from the cell (13, 14). Furthermore, studies from our lab have demonstrated that cell shrinkage and potassium efflux from thymocytes is an early event in apoptosis that allows for the subsequent activation of caspases and nucleases (15).

A primary function of the plasma membrane is the maintenance of a potential difference by its ability to barricade the free passage of ions across the membrane. Normally, most cells maintain an electrical potential across the plasma membrane of −60 mV to −70 mV that renders the inside of the membrane more negative than the outside (16). This is due to the asymmetrical distribution of ions across the plasma membrane (17, 18). In some cellular processes, orchestrated changes in plasma membrane potential may be involved. For example, T cells depolarize during mitogenic activation (19, 20), and these potential changes were accompanied by changes in ion permeability, but the precise role of such changes in cellular homeostasis is poorly understood.

Until recently, the role of plasma membrane potential in apoptosis has been largely ignored; however, recent studies have suggested that the plasma membrane potential may be compromised during apoptosis of lymphocytes (21). For example, in Jurkat cells, three different stimuli that differ in their mode of action, anti-Fas antibody, A23187, and thapsigargin, all induced a loss of plasma membrane potential associated with apoptosis. In the present investigation, we wished to investigate whether primary isolated thymocytes undergo plasma membrane potential changes in response to treatment by glucocorticoids or other steroids and further, to help define the sequence of events that leads to the loss of plasma membrane potential in thymocytes. To accomplish this goal, we used an oxonal dye that has been previously
shown to be selectively sensitive to plasma membrane potential changes in lymphocytes (21, 22). The data presented herein indicate that the loss of plasma membrane potential is a fundamental feature of thymocyte apoptosis and that the ability of glucocorticoids to induce a loss of plasma membrane potential is restricted to their ability to induce apoptosis in the target cell.

Materials and Methods

Reagents

FCS was purchased from Summit Biotechnology (Fort Collins, CO) and dexamethasone was purchased from Steraloids (Wilton, NH). DiBAC$_4$(3) was purchased from Molecular Probes, Inc. (Eugene, OR). Fas-L was purchased from Kamiya Biomedical Co. (Seattle, WA). A23187 was purchased from Calbiochem (La Jolla, CA). Cycloheximide, actinomycin-D, propidium iodide, and thapsigargin were purchased from Sigma (St. Louis, MO). RU486 was a gift of Dr. R. Deraedt, Roussel-Uclaf (Romainville, France).

Animals

Male Sprague Dawley rats (2–3 months of age) were used in all experiments. The animals were bilaterally adrenalectomized by the provider at least 5 days before use and maintained under controlled conditions of temperature (25°C) and lighting and allowed free access to food and 0.85% saline. For each in vivo study, one rat was administrated dexamethasone (5 mg/kg BW, DEX) by ip injection of the steroid re-suspended in PBS by sonication. A control rat received PBS alone (CON). Three independent in vivo experiments were conducted. All experimental protocols were approved by the animal review committee at the institute and were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals published by the Public Health Service. Animals were killed by decapitation and the thymus was surgically removed.

Thymocyte cultures

To expand our analysis of glucocorticoid-induced thymocyte depolarization, we turned to an in vitro model which we have described previously (4). Following surgical removal of the thymus from an untreated adrenalectomized rat, thymocytes were prepared according to previously published methods (4, 23). Thymocytes were dispensed by gentle homogenization in a Kontes no. 22 glass/glass homogenizer (Kontes Co., Vineland, NJ), filtered through 20 μm Nitex mesh (Tetko, New York, NY) washed in cold PBS and counted on a hemacytometer. Cells were cultured at 5 x 10$^5$ cells/ml in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine, 100 U/ml penicillin, and 75 U/ml streptomycin sulfate (prepared by the media facility at NIEHS). Cells were incubated at 37°C, 5% CO$_2$ for 0, 2, 4, and 6 h before harvest.

HeLa cell culture

HeLa S3 cells were grown in suspension culture at 37°C in Joklik’s MEM, supplemented with 2% FCS, 2 mM glutamine, 75 U/ml penicillin, and 50 U/ml streptomycin sulfate (prepared by the media facility at NIEHS).

FACS analysis and analysis of plasma membrane potential

Plasma membrane potential was measured with the anionic oxonal dye, DiBAC$_4$(3). Previous studies from our lab (21) as well as others (22) have established that DiBAC$_4$(3) serves as an indicator for the plasma membrane potential in lymphoid cells. In the resting state, DiBAC$_4$(3) is excluded from the cell. Upon plasma membrane depolarization, DiBAC$_4$(3) enters the cell and can be detected with FACS analysis by the increase in fluorescence. For plasma membrane potential analyses, cells were incubated with 150 nM DiBAC$_4$(3) (Molecular Probes, Inc.) for 30 min at 37°C, 5% CO$_2$. To exclude cells that had lost membrane integrity, propidium iodide was added to a concentration of 10 μg/ml (24). All fluorescence measurements were made with a Becton Dickinson and Co. (San Jose, CA) FACSort equipped with CellQuest software (Becton Dickinson and Co.). Propidium iodide fluorescence was measured on FL-3 (650 nm) to exclude nonviable cells. Cell size was monitored by alterations in the forward light-scattering properties of the cells as described previously (4). To determine membrane potential, the DiBAC$_4$(3) fluorescence of 15,000 viable cells was measured on FL-1 (excitation at 488 nm, emission at 530 nm). An increase in DiBAC$_4$(3) fluorescence indicated a decrease in plasma membrane potential. The percentage of cells with increased DiBAC$_4$(3) fluorescence was determined by gating on the fresh, viable population of cells. Cells with DiBAC$_4$(3) fluorescence greater that that for the fresh population were quantified for each treatment. The averages ± SEM for each treatment represent at least three independent experiments. Statistical analyses were performed using the Student’s t test with α = 0.05. In the text, * indicates that the treatment is significantly different from freshly isolated cells (P < 0.05). ** indicates that the treatment is significantly differently from time-matched control cells (P < 0.05).

Results

In vivo plasma membrane depolarization induced by dexamethasone

In vivo administration of glucocorticoids causes pronounced thymic regression as a result of thymocyte apoptosis. Approximately 90% of the thymocytes are eliminated within 48–72 h of glucocorticoid treatment (25) via biochemical and morphological characteristics that are consistent with cells undergoing apoptosis (26). To determine whether glucocorticoids alter plasma membrane potential, thymocytes were isolated from rats 4 h after an ip injection of dexamethasone (5 mg/kg BW) as described in Materials and Methods. Freshly isolated thymocytes from control rats had low DiBAC$_4$(3) fluorescence (11.0 ± 1.4%), indicating that the plasma membrane potential was intact and that the cells can exclude the dye (Fig. 1). In vivo treatment with dexamethasone produced a population of viable cells with increased DiBAC$_4$(3) fluorescence that was significantly larger than the control population (25.9 ± 3.4% **). This increase in DiBAC$_4$(3) fluorescence indicates that the cells have lost plasma membrane potential. Thus, these results show that in vivo glucocorticoid treatment results in an early depolarization of thymocytes before the loss of membrane integrity.

Time course for the loss of plasma membrane potential in primary isolated rat thymocytes

To extend our observation that glucocorticoids depolarize thymocytes in vivo and to determine whether this is a direct effect on thymocytes or an indirect action of glucocorticoids, we cultured primary isolated thymocytes and evaluated the ability of glucocorticoids to depolarize thymocytes in vitro. Previous studies from our lab have demonstrated that primary isolated rat thymocytes undergo a spontaneous death program in culture that is accelerated by the addition of glucocorticoids (4, 27). First, to establish a time course for the loss of plasma membrane potential in rat thymocytes during apoptosis, thymocytes were cultured for the specified times in the presence or absence of dexamethasone. Following the treatment period, cells were analyzed by flow cytometry to evaluate plasma membrane potential as described above. Following 6 h of culture in the absence of dexamethasone, the control cells have a population of cells with increased DiBAC$_4$(3) fluorescence (16.9 ± 1.8%) compared with
freshly isolated cells (8.5 ± 1.8%), indicating a loss of plasma membrane potential (Fig. 2). Dexamethasone treatment results in a significantly larger population of cells with an increase in DiBAC4(3) fluorescence (40.7 ± 3.9% **). This is consistent with the fact that dexamethasone increases the percentage of cells undergoing apoptosis and demonstrates that dexamethasone also increases the percentage of cells with a depolarized plasma membrane. It is important to note that glucocorticoid treatment increases the number of cells that depolarize, but not the magnitude of depolarization.

**Dexamethasone induces a loss of plasma membrane potential in a dose-dependent manner**

Previous studies from our lab have established that dexamethasone induces cell death and caspase-3-like activity in rat thymocytes at concentrations that reflect its affinity for the glucocorticoid receptor (4). To determine whether the dexamethasone-induced loss of plasma membrane potential is dose-dependent, primary isolated rat thymocytes were analyzed for a loss of plasma membrane potential after 6 h in culture. Treatment with dexamethasone induced a dose-dependent increase in the percentage of cells with higher DiBAC4(3) fluorescence (Fig. 3). The maximum effect occurred at a dexamethasone concentration of 100 nM, with 45.6 ± 5.7% (**) of the cells depolarized, compared with 18.4 ± 1.9% (*) of the cells depolarized in the spontaneously dying population and 11.2 ± 2.1% of the cells in the fresh population. Thus, dexamethasone depolarizes rat thymocytes in a dose-dependent fashion.

**Steroid specificity of the loss of plasma membrane potential in rat thymocytes**

We next evaluated the steroid specificity of dexamethasone-induced loss of plasma membrane potential in rat thymocytes. First, we compared the ability of two natural glucocorticoids, cortisol and corticosterone to induce a loss of plasma membrane potential in rat thymocytes. Primary isolated thymocytes were cultured for 6 h with 100 nM dexamethasone, 100 nM cortisol, or 100 nM corticosterone. Both cortisol (40.4 ± 5.1% **) and corticosterone (38.0 ± 3.4% **) induced a loss of plasma membrane potential comparable to dexamethasone (40.5 ± 3.5% **) (Fig. 4A). This demonstrates that the loss of plasma membrane potential can be induced by other glucocorticoids besides dexamethasone. To determine whether the ability to induce a loss of plasma membrane potential in primary rat thymocytes extends to other
classes of steroids, primary thymocytes were treated with 1 μM 17β-estradiol, progesterone, or dihydrotestosterone for 6 h. The percentage of cells with increased DiBAC₄(3) fluorescence for 17β-estradiol, progesterone, and dihydrotestosterone were, 20.4 ± 3.3%, 22.5 ± 1.1%, and 16.2 ± 0.5%, respectively. Thus, while the percentage of cells with increased DiBAC₄(3) fluorescence increased above fresh levels (6.25 ± 0.46%) none of these steroids significantly induced a loss of plasma membrane potential above spontaneous levels (16.6 ± 1.05% * ) (Fig. 4B). In addition, the percentage of cells with increased DiBAC₄(3) fluorescence was significantly lower following treatment with these steroid hormones compared with dexamethasone. These data demonstrate that the ability of glucocorticoids to induce a loss of plasma membrane potential is unique to glucocorticoids and does not extend to the other classes of steroid hormones evaluated in this study despite the fact that these hormones are known to regulate thymic function (28–30). These results are consistent with our previous findings that glucocorticoids selectively activated caspases and induced apoptosis in primary thymocytes (4, 27).

Other apoptotic stimuli depolarize the plasma membrane of primary rat thymocytes

The observation that the loss of plasma membrane potential in rat thymocytes occurs in response to glucocorticoids and not to other steroids, raised the question of whether or not other nonsteroid apoptotic signals can depolarize primary thymocytes. To address this question, we compared the ability of three other apoptotic agents, each of which induces apoptosis by a separate mechanism, to depolarize primary thymocytes. Fas-L, which induces apoptosis through its interaction with the Fas receptor, increased the percentage of cells with DiBAC₄(3) fluorescence compared with the time-matched control (Fig. 5). A similar effect was observed with two additional compounds known to induce apoptosis in T cells, the calcium ionophore A23187(1) and the Ca²⁺-ATPase inhibitor thapsigargin (31). Together, these results demonstrate that other apoptotic agents besides glucocorticoids can depolarize the plasma membrane of thymocytes. In addition, this supports our previous observation that Fas receptor signaling, A23187, and thapsigargin can depolarize Jurkat cells (21) and implies an important role for plasma membrane depolarization in apoptosis.

Glucocorticoids induced a loss of plasma membrane potential only in cells that undergo apoptosis

The fact that other apoptotic agents besides glucocorticoids can depolarize thymocytes suggests that the ability of glucocorticoids to depolarize the plasma membrane correlates with their ability to induce cell death. To address this issue, we determined whether cells that are sensitive to glucocorticoids but do not undergo glucocorticoid-induced apoptosis lose plasma membrane potential in response to glucocorticoid treatment. Previous studies have established that dexamethasone treatment of HeLa cells results in regulation of the functional response to steroid hormone, including translocation of the glucocorticoid receptor to the nucleus.
(32), glucocorticoid receptor down-regulation (33), and regulation of alkaline phosphatase activity (34). Glucocorticoid treatment, however, does not induce apoptosis in HeLa cells. In this study, when HeLa cells are treated with 100 nM dexamethasone for 6 h, we observe no difference in DiBAC 4 (3) fluorescence between control and dexamethasone-treated populations (Fig. 6). In contrast, dexamethasone causes a substantial loss of plasma membrane potential in rat thymocytes, as evidenced by the dramatic increase in DiBAC4 (3) fluorescence. These results suggest that the ability of glucocorticoids to induce a loss of plasma membrane potential correlates with their ability to induce apoptosis.

Glucocorticoid-induced loss of plasma membrane potential is dependent upon its interaction with the glucocorticoid receptor

Glucocorticoids induce apoptosis in primary thymocytes through their interaction with the glucocorticoid receptor. The receptor antagonist RU486, which causes glucocorticoid receptor translocation (35) and repression of NF-κB (36), but does not transactivate glucocorticoid-regulated genes (35), does not induce apoptosis. We have previously shown that RU486 also blocks glucocorticoid-induced cell shrinkage but does not block spontaneous shrinkage in thymocytes (4). To further explore the mechanisms behind glucocorticoid-induced depolarization of thymocytes and its role in apoptosis, we investigated the effects of the glucocorticoid receptor antagonist RU486 by simultaneously comparing depolarization and cell shrinkage. As we have seen previously, RU486 alone did not protect or enhance spontaneous shrinkage in HeLa cells. In addition, RU486 alone did not significantly increase DiBAC 4 (3) fluorescence over spontaneous levels (21.5 ± 1.8% * vs. 16.9 ± 1.4% *, respectively), suggesting that the depolarization induced by the glucocorticoid receptor requires interaction with the agonist ligand (Fig. 7). Conversely, RU486 did not block the loss of plasma membrane potential associated with spontaneous cell death, as the percentage of depolarized cells in the RU486 treatment was significantly different from freshly isolated cells (8.5 ± 1.8%). These observations correlate with the fact that RU486 does not alter the forward light-scattering properties of the cell compared with the spontaneously dying population.

The receptor antagonist RU486 did block the loss of plasma membrane potential associated with dexamethasone-induced cell death to the same level as spontaneously dying cells. Although dexamethasone increased the percentage of depolarized cells to 40.7 ± 3.9% **, RU486 blocked this increase to 23.1 ± 2.8% *, which is equivalent to the number of depolarized cells seen in thymocytes treated with RU486 alone. These data indicate that although the spontaneous loss of plasma membrane potential is not dependent upon the glucocorticoid receptor, glucocorticoid-induced loss of plasma membrane potential is dependent upon the glucocorticoid receptor. These data also demonstrate that depolarization is limited to the shrunken population of cells. RU486, in addition to blocking depolarization, also blocked dexamethasone-induced shrinkage, as we have observed previously (4), and suggests a close relationship between depo-

![Fig. 5. Other apoptotic stimuli induce plasma membrane depolarization in thymocytes. Primary thymocytes were cultured for 6 h in the presence of different apoptotic agents to evaluated their ability to depolarize thymocytes. Cells were stained with DiBAC 4(3) as described in Materials and Methods. Propidium iodide was added before flow cytometric analysis, and viable cells were analyzed by flow cytometry for DiBAC 4(3) fluorescence. The figure shows representative DiBAC 4(3) fluorescence histograms for untreated thymocytes (gray line) or thymocytes treated with 100 nM dexamethasone, 1 ng/ml fas-L, 2 μM A23 187, or 0.5 μM thapsigargin (solid black).](https://academic.oup.com/endo/article-abstract/142/1/421/2989173)
larization and shrinkage in this model system. This provides further support for a role for depolarization in glucocorticoid-induced apoptosis.

**Glucocorticoid-induced loss of plasma membrane potential is dependent upon de novo gene expression**

It is well established that glucocorticoid-induced apoptosis of rat thymocytes is dependent upon *de novo* gene expression (37, 38). The manifestation of biochemical endpoints of apoptosis, including phosphatidylserine exposure, cell shrinkage, and caspase activation, are all dependent upon *de novo* gene expression (4). Spontaneous apoptosis of thymocytes, however, occurs independently of *de novo* gene expression. To determine whether the loss of plasma membrane potential is dependent upon *de novo* gene expression in primary rat thymocytes, thymocytes were cultured with actinomycin-D to prevent RNA synthesis or cycloheximide to prevent protein synthesis. Spontaneously dying thymocytes showed a loss of plasma membrane potential independent of either RNA or protein synthesis (Fig. 8). Inhibition of protein synthesis by cycloheximide or inhibition of RNA synthesis by actinomycin-D significantly increased the percentage of depolarized cells over freshly isolated thymocytes to 18.7 ± 1.7% * and 14.6 ± 2.7% *, respectively. However, these values were not significantly different from spontaneously dying thymocytes, indicating that at this time point, inhibition of protein or RNA synthesis neither potentiates nor inhibits the depolarization of the plasma membrane in spontaneously dying cells. These data also confirmed that the failure of protein and RNA synthesis inhibition to affect spontaneous depolarization correlated with the inability to enhance or prevent spontaneous cell shrinkage.

Glucocorticoid-induced loss of plasma membrane potential, in contrast to the spontaneous loss of plasma membrane potential, required *de novo* gene expression. The appearance of the depolarized population of cells in the glucocorticoid treatment was blocked by cycloheximide and actinomycin-D to the same levels as the time-matched controls (20.1 ± 3.0% * and 19.7 ± 2.6% *, respectively). In addition, depolarization was again limited to the shrunken population of cells and inhibition of gene expression simultaneously blocked both depolarization and shrinkage. These results are consistent with our previous observations that glucocorticoid-induced but not spontaneous apoptosis requires *de novo* gene expression (4). In addition, these results place depolarization downstream of gene expression along with cell shrinkage in the glucocorticoid-induced apoptotic pathway of primary thymocytes.

**Discussion**

It is well documented that glucocorticoids affect the structure and function of the thymocyte plasma membrane during glucocorticoid-induced apoptosis by inducing membrane blebbing, reorientation of phosphatidylserine residues, and by altering the transmembrane transport of amino acids, glucose, and nucleosides (7–10). In this study, we have established that physiological concentrations of glucocorticoids can also depolarize the plasma membrane of thymo-
cytes and that this correlates with their ability to induce apoptosis in thymocytes. Our observations are supported by an earlier study that observed depolarization at pharmacological doses of methylprednisolone (39). The loss of plasma membrane potential precedes the loss of membrane integrity, as measured by the ability of the cells to exclude propidium iodide. The percentage of viable cells with a loss of plasma membrane potential increased as the length of time of exposure to the apoptotic stimulus increased. Glucocorticoids and other stimuli increased the number of depolarized cells, but not the magnitude of depolarization. This was observed both with survival factor withdrawal and glucocorticoid-induced apoptosis. A similar effect has been observed with other markers of apoptosis (4). The percentage of depolarized cells in a dose-dependent fashion that reflects the binding of glucocorticoids to the glucocorticoid receptor. We have also observed that Jurkat cells treated with increasing doses of anti-Fas antibody also show a dose-dependent increase in the percentage of depolarized cells (21). Thus, the percentage of depolarized thymocytes corresponds to the duration and intensity of exposure to glucocorticoids.

The ability of glucocorticoids to induce thymocyte apoptosis is unique to this class of steroids. We have previously shown that the activation of caspase-3-like activity, a fundamental feature of glucocorticoid-induced apoptosis in thymocytes, is unique to glucocorticoids (4). In the present study, the ability of glucocorticoids to induce a loss of plasma membrane potential was also unique to this class of steroids. Although cortisol and corticosterone depolarized the plasma membrane to a similar degree as dexamethasone, extremely high doses of other steroid hormones did not increase the percentage of depolarized cells above control levels. Although other steroid hormones could not depolarize thymo-

Fig. 7. Glucocorticoid-induced apoptosis is dependent upon the interaction with the glucocorticoid receptor. Cell size and plasma membrane potential were examined simultaneously in primary thymocytes cultured alone (CON) or in the presence of 1 μM RU486, 100 nM dexamethasone, or both. Cells were stained with DiBAC$_4$(3) as described in Materials and Methods. Propidium iodide was added before flow cytometric analysis, and viable cells were analyzed by flow cytometry. To compare DiBAC$_4$(3) fluorescence vs. cell size, cells were analyzed on a representative DiBAC$_4$(3) fluorescence vs. forward scatter plot.
cytes, we found that other apoptotic stimuli can. A nonste-
roidal apoptotic agent, Fas-L induced a rapid loss of plasma
membrane potential in the primary thymocytes, as did two
other apoptotic agents, A23187 and thapsigargin. This sup-
ports previous findings from our laboratory which show that
anti-Fas antibody, A23187, and thapsigargin can depolarize
Jurkat cells (21). In addition, this observation suggests that
the ability of glucocorticoids to depolarize thymocytes is
related to their ability to induce apoptosis.

Glucocorticoids induce tissue specific changes depending
on the target tissue. In the case of immune cells, glucocor-
ticoids trigger apoptosis. This cell death program is charac-
terized by the classical morphologic and biochemical
changes traditionally associated with apoptosis such as cell
shrinkage and internucleosomal DNA fragmentation. Al-
though plasma membrane depolarization has been recently
described in apoptosis, it was unknown whether the plasma
membrane depolarization induced by glucocorticoids was a
general effect regardless of the target cell, or whether it was
a specific effect associated with apoptosis. HeLa cells, which
display nuclear translocation of ligand-bound glucocorticoid
receptor (32), but do not undergo apoptosis, failed to depo-
larize in response to glucocorticoid treatment. These data
clearly show that the ability of glucocorticoids to induce
plasma membrane depolarization in the target cell is directly
related to their ability to induce apoptosis.

The fact that inhibition of glucocorticoid receptor binding
and de novo gene expression did not block the spontaneous
loss of plasma membrane potential demonstrates that in
survival factor withdrawal-induced death, the glucocorti-
coid receptor and gene regulation are not required for plasma
membrane depolarization. These data support our previous
observation that the biochemical features of spontaneous cell
death manifest independently of the glucocorticoid receptor
and de novo gene expression (4). These experiments also
demonstrated that the glucocorticoid receptor requires in-
teraction with the agonist ligand to induce cellular depolar-
ization. In fact, we also observe that the glucocorticoid an-
tagonist RU486 can block the simultaneous depolarization
and shrinkage caused by glucocorticoids, as can inhibition of
gene expression. These results are consistent with previous
studies, which show that the glucocorticoid receptor and
subsequent gene regulation are required for glucocorticoid-
induced cell shrinkage, phosphatidylserine flipping, and
other endpoints of apoptosis. The fact that depolarization is
limited to the shrunken population of cells and that agents
that block cell shrinkage in thymocytes also block depolar-
ization lends further proof that plasma membrane depolar-
ization is an important component of the apoptotic pathway.

Plasma membrane depolarization is an essential compo-
nent of many cellular processes in a variety of cell types.
Stimulus-induced alterations to the electrical field across a

![Fig. 8. Glucocorticoid-induced depolarization is dependent upon de novo gene expression. Cell size and plasma membrane potential were examined simultaneously in primary thymocytes cultured for 6 h alone (CON) or in the presence of cycloheximide (10 μM), actinomycin-D (1 μg/ml), or dexamethasone (100 nM). Cells were stained with DiBAC₄(3) as described in Materials and Methods. Propidium iodide was added before flow cytometric analysis, and viable cells were analyzed by flow cytometry. To compare DiBAC₄(3) fluorescence vs. cell size, cells were analyzed on a representative DiBAC₄(3) fluorescence vs. scatter plot. To inhibit protein synthesis, primary thymocytes were cultured with cycloheximide (1 μM) in the presence or absence of dexamethasone for 6 h. To inhibit RNA synthesis, primary thymocytes were cultured with actinomycin-D (1 μg/ml) in the presence of absence of dexamethasone for 6 h.](https://academic.oup.com/endo/article-abstract/142/1/421/2989173)
membrane could effect the structure and function of charged, membrane-embedded macromolecules. Indeed, conformational changes in voltage-dependent ion channels of electrically excitable cells are mediated by changes in the plasma membrane potential (40, 41). Stimulus-secretion coupling in some nonexcitable cells is also mediated by ionic and electrical events at the plasma membrane (42). In lymphocytes, plasma membrane depolarization plays a central role in lymphocyte activation. Activation of T cells by antigen-dependent or antigen-independent stimuli results in plasma membrane depolarization, turnover of polyphosphoinositides, and an increase in free intracellular calcium (20). In the present study, we have described the effect of glucocorticoids on plasma membrane potential during thymocyte apoptosis and define a sequence of events that places the loss of plasma membrane potential early in the apoptotic pathway following gene expression. Currently, the specific mechanism that leads to apoptotic plasma membrane depolarization and the exact consequences of this depolarization are unclear. However, studies to identify specific targets involved in glucocorticoid-induced loss of plasma membrane potential are currently underway in our laboratory.

Acknowledgments

We would like to thank Carl D. Bortner for his technical assistance and Sue Edelstein of Image Associates, Inc. for her assistance with graphic design.

References