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Mitochondrial Membrane Potential Changes in Osteoblasts Treated with Parathyroid Hormone and Estradiol

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This study assessed mitochondrial membrane potential changes in cultured osteoblasts treated with hormones known to regulate osteoblasts. A fluorescent carbocyanine dye, 5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolocarbocyanine iodide, also called JC-1, was used as a probe. JC-1 emits photons at 585 nm (orange–red) when the membrane potential in mitochondria is highly negative, but when the potential becomes reduced emission occurs at 527 nm (green). Osteoblasts were rinsed in serum-free medium for 5 min, then loaded with 1×10^{-6} *M* JC-1 for 10 min. The distribution and intensity of JC-1 fluorescence were evaluated with a laser-scanning confocal microscope system. Hormone treatments included parathyroid hormone (PTH; 10^{-8} *M*), 17β-estradiol (10^{-8} *M*), and thyroxine (T_4; 10^{-8} *M*). The potassium ionophore valinomycin (10^{-6} *M*) was used as a control since it is known to disrupt the electrochemical gradient of mitochondria without interfering with the pH gradient. Valinomycin caused a profound, rapid increase (22.5% above untreated values) in the green/red ratio, which indicated a lowering of the mitochondrial membrane potential in all samples evaluated. PTH caused a less pronounced, but significant (7–14%), reduction in membrane potential in all cells examined. PTH is known to affect osteoblasts in a number of ways and is inhibitory to mitochondrial respiration; the results confirm this effect. For estradiol, half of the cells responded at a significant level, with a membrane potential reduction of 6 to 13% being recorded; the other half did not respond. Thyroxine did not alter mitochondrial membrane potential. Responses were detectable within 20 s for valinomycin, but occurred at a slower rate, over 200 to 300 s, following PTH and estradiol treatment. Responses to PTH and estradiol could be due to mitochondrial uptake of cytosolic Ca^{2+}.

INTRODUCTION

The ability to study and understand the cell biology of bone took a major step forward when it became possible to culture bone cells either as primary cells or as cell lines. This technology has made it possible to evaluate many aspects of bone cell function. Another useful advance is the development of fluorescent probes that allow noninvasive studies of cell metabolism to be carried out on individual cells.

Osteoblasts have three major roles: to synthesize and secrete a calcifiable matrix, to orchestrate calcification of the matrix, and to participate in the regulation of bone remodeling by interacting with osteoclasts, the cells that resorb bone. Typical of cells which synthesize protein, osteoblasts contain a substantial amount of endoplasmic reticulum, as well as Golgi vesicles. Mitochondria are present in moderate amounts and are dispersed throughout the cytoplasm, but are especially abundant in the Golgi region [1].

As defined by Mitchell [2], mitochondria generate a proton gradient across their inner membranes by proton pumping. The gradient, which consists of an electric component (the membrane potential) and a chemical component (a pH gradient), supports a high rate of ATP synthesis. The potential can be as high as 240 mV across the 5-nm-thick membrane [3]. The mitochondrion is the only intracellular organelle that has such a substantial negative membrane potential. The high negativity is located in the matrix space which is defined by the inner mitochondrial membrane. The membrane potential also supports other functions such as uptake of Ca^{2+}, maintenance of mitochondrial protein synthesis, and importation of mitochondrial enzymes [4, 5].

Because of high internal negativity, lipophilic compounds with a delocalized positive charge will be taken up by mitochondria to a greater extent than by other organelles, including the plasma membrane. The Nernst equation predicts that such a compound would become more concentrated in the more electronegative mitochondria than beneath the plasma membrane. Before reaching equilibrium inside the cell most of the lipophilic cations would be drawn into the more negative mitochondria rather than into the cytoplasm or other organelles. Lipophilic cations that are also fluorescent have been designed to serve as reporter mole-

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cules for monitoring mitochondrial activity. The classes of such fluorescent probes include carbocyanine, acridine, pyrilium, and quinolinium dyes and various members of the rhodamine family. All of these are lipophilic and have delocalized positive charges and consequently will accumulate in and brightly stain mitochondria in accordance with the Nernst potential [6].

The lipophilic cation of interest in the present investigation is the carbocyanine dye, 5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolocarbocyanine iodide, commonly referred to as JC-1. This molecule, discovered by and named after its discoverer E. E. Jelley [7], exists as a monomer at low concentrations and as aggregates, called J-aggregates, at high concentrations. JC-1 monomer is excited at 490 nm and emits at around 527 nm. Upon aggregation, fluorescence emission shifts to 585 nm [5, 8]. As a consequence, mitochondria having low membrane potential will accumulate low concentrations of JC-1 and will fluoresce in green; in more highly polarized regions within mitochondria fluorescence will be orange-red. Chen and Smiley [5] estimate that mitochondria with membrane potentials exceeding 140 mV would be capable of forming J-aggregates. These properties make JC-1 a more useful probe than the other lipophilic cations for assessing mitochondrial function. When, for example, cells are stained with rhodamine-123, mitochondria tend to stain intensely regardless of membrane potential [5, 9].

In the present study, we evaluated the effects of two hormones known to alter osteoblast function, namely parathyroid hormone and estrogen. Parathyroid hormone (PTH) has several systemic effects. Of interest here is that it acts directly on osteoblasts [10]. PTH has been reported to reduce the rate of mitochondrial respiration in kidney and heart tissue by uncoupling oxidative phosphorylation [11, 12]. PTH has also been shown to bind directly to the β-subunit of mitochondrial F_1 ATPase and in so doing may act as an intracellular receptor that mediates the effects of the hormone [13]. However, in the latter study it was not clear how an extracellular peptide could access a subcellular compartment. One intracellular signal of plasma membrane receptor-bound PTH has been shown to be Ca^{2+} [14].

Estrogen has been established as binding to nuclei and influencing proliferation of osteoblasts [15–20]. More recently, 17β-estradiol bound to BSA, and consequently unable to enter cells, has been found to stimulate release of Ca^{2+} from intracellular stores in isolated rat osteoblasts [21].

Thyroxine was selected as a test substance because of its ability to increase basal metabolism in many cell types. While the better understood action of thyroid hormone is to alter gene expression [22], thyroid hormones are also known to bind to plasma membrane receptors and cause an increase in the rate of respiration in mitochondria [23].

Valinomycin is a potassium ionophore that can be used to dissipate mitochondrial membrane potential without affecting the pH gradient [4, 6]. Consequently, valinomycin was used as a control to verify that the fluorescent structures observed were mitochondria and that dissipation of membrane potential resulted in an increased green/red fluorescence ratio.

In this study, we show that valinomycin and parathyroid hormone dissipate the membrane potential of osteoblast mitochondria in all cells examined; estrogen had a milder membrane potential lowering effect on approximately half of the cells examined; and thyroxine was ineffective.

MATERIALS AND METHODS

Osteoblast isolation. Day-old Peterson X Arbor Acre male chickens were obtained from Metz Hatchery (Belleville, PA) and raised for 2–3 weeks at the Penn State Poultry Education and Research Center on a normal chick starter diet. The birds were decapitated, an I.A.C.U.C. and American Veterinary Association approved procedure. The tibias were removed aseptically and cleaned of adherent tissue and periosteum using sterile gauze. Excised tibia were subjected to a sequential mild enzymatic digestion procedure to remove osteoblasts from the periosteal surface as previously described [24]. Osteoblasts were maintained in phenol red free Dulbecco's modified Eagle's medium (DME), formulation D-2902, to which 3.7 g/liter sodium bicarbonate, 0.05 g/liter ascorbate, 100 U penicillin–100 μg streptomycin, and 10% heat inactivated fetal bovine serum were added (Sigma, St. Louis, MO). The pH was adjusted to 7.1. Half of the culture medium was replaced every 2–3 days. Experiments were conducted on 1-week-old cells.

Image acquisition by confocal microscopy. Osteoblasts on coverslips were rinsed with serum-free DME (37°C, 5 min) and mounted in an RC-21B perfusion chamber (Warner Instruments, Hamden, NJ) which contained serum-free DME plus 1×10^{-6} *M* JC-1 (5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenimidazolocarbocyanine iodide) from Molecular Probes, Eugene, OR. The chamber, originally designed to accept 22-mm square glass coverslips, was modified to accept 12-mm round coverslips. A coverslip with adherent osteoblasts was inverted and fitted into the chamber. The chamber was mounted onto the microscope stage and polyethylene tubing (ID 0.045 in, OD 0.062 in) attached to the chamber entry port. Cells were then viewed in the Bio-Rad MRC 600 laser-scanning confocal imaging system equipped with a Krypton–Argon laser and interfaced with a Leitz microscope. A neutral density filter was used to allow 10% transmittance of the laser beam. The direct filter and fast scan rate were used. The fast scan option utilized every other laser scan line to reduce exposure of the sample to the laser beam and minimize bleaching effects. Selection of the direct filter setting allowed direct image acquisition without computer enhancement. The K1/K2 filter block set was used to separate emissions into red and green bands. Photomultipliers and aperture size were set to produce the brightest image possible against minimal background. Aperture settings, which control the degree of confocality, were set for both photomultipliers at 12 units. Cells were viewed at room temperature in a darkened room. Analysis was done utilizing the COMOS software package MPL program provided by Bio-Rad (Melville, NY), on a single optical section through each sample. For each sample the position of the optical section was chosen by illuminating the specimen, focusing up and down using the 1-μm-interval step function. An optical

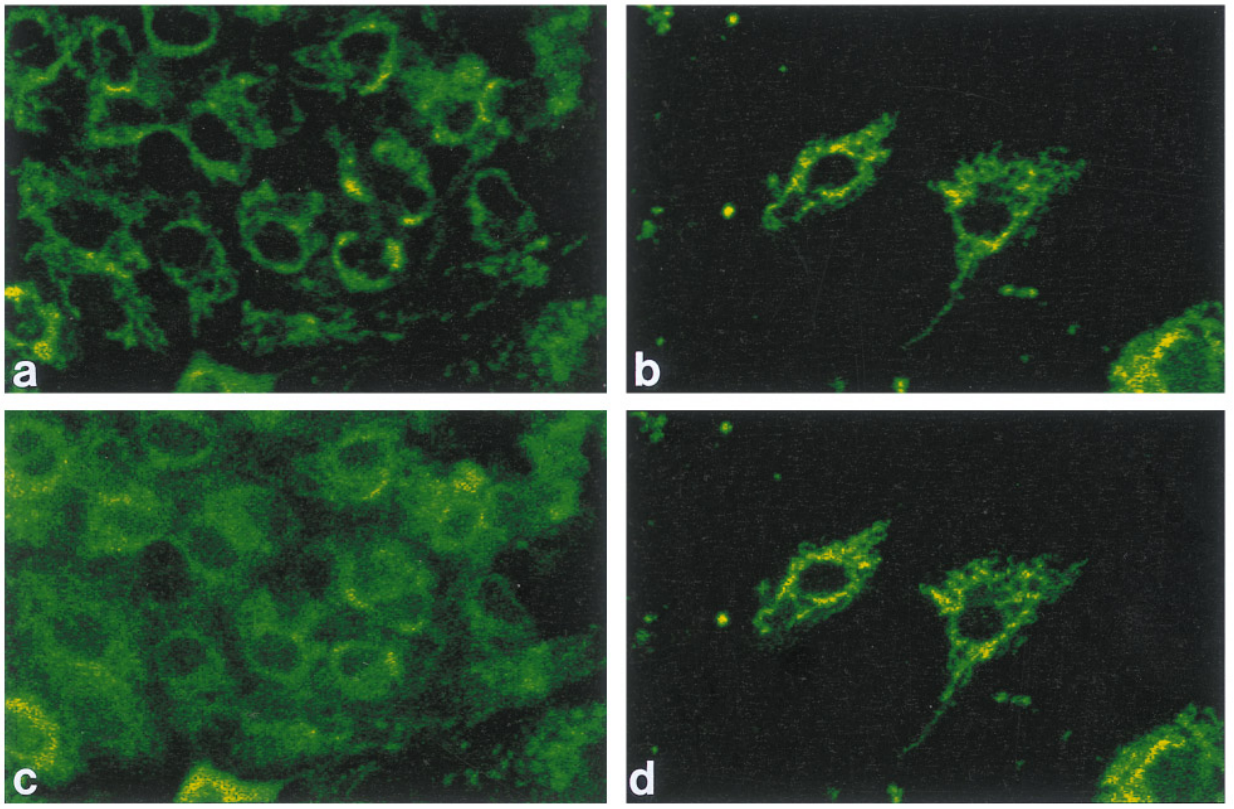

FIG. 1. Typical confocal images of osteoblasts stained with JC-1 (a,b) prior to treatment and after (c) valinomycin treatment for 200 s or (d) PTH treatment for 280 s., Magnification, 600×.

section close to the middle of the cells in the xy-plane was selected for analysis. After establishing baseline fluorescence, a treatment solution was introduced into the chamber. The procedure to this point typically required 5–7 min.

Treatments. Solutions of 1×10^{-8} *M* 17β-estradiol (Sigma), 1×10^{-8} *M* thyroxine (Sigma), 1×10^{-8} *M* parathyroid hormone (bovine, 1-84; National Hormone and Pituitary Program, NIH), and 1×10^{-6} *M* valinomycin (Sigma), were made from stock solutions immediately after the chamber was secured to the microscope stage. Stock solutions of 17β-estradiol, thyroxine, and valinomycin were prepared in ethanol. PTH was initially diluted in 0.9% NaCl and 0.1% bovine serum albumin. Each treatment solution also contained 1×10^{-6} *M* JC-1 made from a stock solution of 1×10^{-2} *M* JC-1 in DMSO. Subsequent dilutions for all solutions were made with serum-free DME. Final solutions contained less than 0.1% ethanol and 0.1% DMSO. Perfusion of test solutions and vehicle into the cell chamber was carefully done to completely push the holding solution from the chamber. The focus motor was deactivated in order to minimize changes in the plane of focus. Typically three cells were analyzed in a field of view and each hormone was tested on cells derived from two to four different cell isolations. Significant differences between treatment and control averages were determined by Student's *t* test.

RESULTS

The distribution of mitochondria in cultured osteoblasts stained by JC-1 is shown in Figs. 1a and 1b. The staining pattern observed reveals the cytoplasm as being packed with thread-like and granular mitochondria. No nuclear staining was evident. Unstained cells were not visible even when observed with maximum sensitivity settings. Background staining was negligible. The addition of valinomycin enhanced green fluorescence for most mitochondria in the plane of view (Fig. 1c). Following valinomycin treatment there was a loss of clarity of the image due to the defined edges of the mitochondrion becoming less distinct and to slight diffusion of the dye out of the cells. This effect did not occur with the addition of the other substances being tested. The image of cells treated with PTH is shown in Fig. 1d; a minor enhancement of green fluorescence was evident by visual inspection. All cells in the field of view responded to PTH (10–30 cells per field of view; 9 fields of view). Responses to estradiol or thyroxine were not apparent by visual inspection of the images (not shown). Shifts in the intensity and pattern of red fluorescence were also difficult to evaluate by visual inspection and therefore are not shown. However, ratiometric analysis revealed consistent changes following all treatments except thyroxine.

Ratiometric analyses were carried out on osteoblasts over selected periods of time, 10 min or less, to quantify

the responses. A shift to more green fluorescence following valinomycin treatment (Fig. 2A) was detectable within 20 s. The magnitude of the shift expressed in the percentage change in ratios for cells treated with valinomycin relative to untreated cells was 22.5% (Table 1). PTH caused a shift in the green/red ratios of all cells examined (Fig. 2B). However, the response to PTH was gradual over a several minute time frame; the magnitude of the change was 14% (Table 1). Statistical analysis demonstrated that the PTH response was significant, highly so at the longer (300 s) treatment time. Estrogen treatment (Fig. 2C) also resulted in a gradual response in some cells that was similar in magnitude to the PTH response. Approximately 50% of estrogen-treated cells responded to a significant or highly significant degree (Table 1). Thyroxine had little or no effect (Fig. 2D and Table 1).

In all cases, cells treated with vehicle over a 10-min time frame were unresponsive (Table 1). Cells not stained with JC-1 were not discernible when illuminated with the laser, indicating that autofluorescence did not confound the data.

DISCUSSION

Confocal microscopy provides a powerful approach for evaluating cell function as a result of its noninvasive optical sectioning property and because the focused image is converted to an electronic signal which allows computer-driven data-processing techniques to be employed. Fluorescent probes can be used both as markers of intracellular molecules and to follow changes in metabolic processes. Because both a point source of nearly monochromatic light and a point detector are utilized, resolution is improved about 1.4-fold over conventional fluorescent microscopy [25]. To obtain meaningful data when using confocal microscopy the specimen must be stabilized so that a narrow plane of focus can be maintained throughout an experiment. We found that even small changes in internal pressure of the cell chamber were sufficient to cause alterations in the plane of focus. The small downward deflection observed immediately following injection of treatment solutions (Fig. 2) is believed to reflect this momentary change in focus.

The extent of autofluorescence was assessed because previous experience in this laboratory has shown that cultured osteoblasts exhibit a greenish autofluorescence in both cells and background when viewed under broad band UV illumination. Also, osteoblasts sometimes contain green autofluorescent granules in the cytoplasm. Autofluorescence was ruled out as a source of photons that could confound the data when a narrow range of wavelengths was employed to generate fluorescence. A series of background control measurements revealed that at maximal sensitivity settings, unstained cells were not discernible.

When cells were frequently scanned with 5 or 10 s between scans, fluorescence diminished to an undetectable level. This pronounced photobleaching effect was corrected by using a 10% neutral density filter and scanning at 20-s intervals. These maneuvers resulted in green/red ratios that were stable over the experimental time frame. The stabilizing conditions are believed to be the consequence of the cells being bathed in JC-1 solution at all times where unbleached monomer units could then diffuse into a region and reach equilibrium with bleached monomer which would diffuse away from the small area of the coverslip being scanned.

Valinomycin is a potassium ionophore that is known to destroy mitochondrial membrane potential without interfering with the proton gradient [4, 6]. One would expect that such a compound would cause a shift away from a large negative membrane potential, which is characterized by red fluorescence, toward a lower membrane potential revealed by green fluorescence; such a shift was observed. This response corresponds to results reported for two human cell lines, U937 and K562 [26]. The response to valinomycin indicates that the staining observed was specifically in mitochondria and not in another organelle.

Parathyroid hormone treatment resulted in a trend similar to that seen for valinomycin, i.e., a decrease in mitochondrial membrane potential, but the decrease was not as pronounced or as rapid. PTH has a multiplicity of effects on osteoblasts, as recent reviews indicate [27, 28]. Of particular relevance to this study are reports of effects on mitochondria in PTH-responsive cells. The influence of PTH on isolated mitochondria has been known for many years with effects ranging from activation of ATPase, enhanced hydroxylation of 25-OH vitamin D_3, increased substrate oxidation, and uncoupling oxidative phosphorylation [13]. As discussed by Laethum and Zull [13], specific binding sites for PTH have been identified in isolated mitochondria, but the physiological significance of these observations has been questioned because of barriers to internalization created by the plasma membrane to peptide hormones. On the other hand, autoradiographic studies have revealed radiolabeled PTH in sections of renal tissue following injection of the hormone into intact animals [29, 30]. The route of entry of the peptide into the renal cells is believed to be receptor-mediated endocytosis. Internalization of plasma membrane-bound PTH from osteoclast surfaces has been found to occur within 20 min, a process which is also believed to be due to endocytosis [31].

Another means by which PTH may influence mitochondria within intact cells is through increased mitochondrial uptake of Ca^{2+} due to a surge in cytosolic Ca^{2+} concentration. Ca^{2+} is a known intracellular sig-

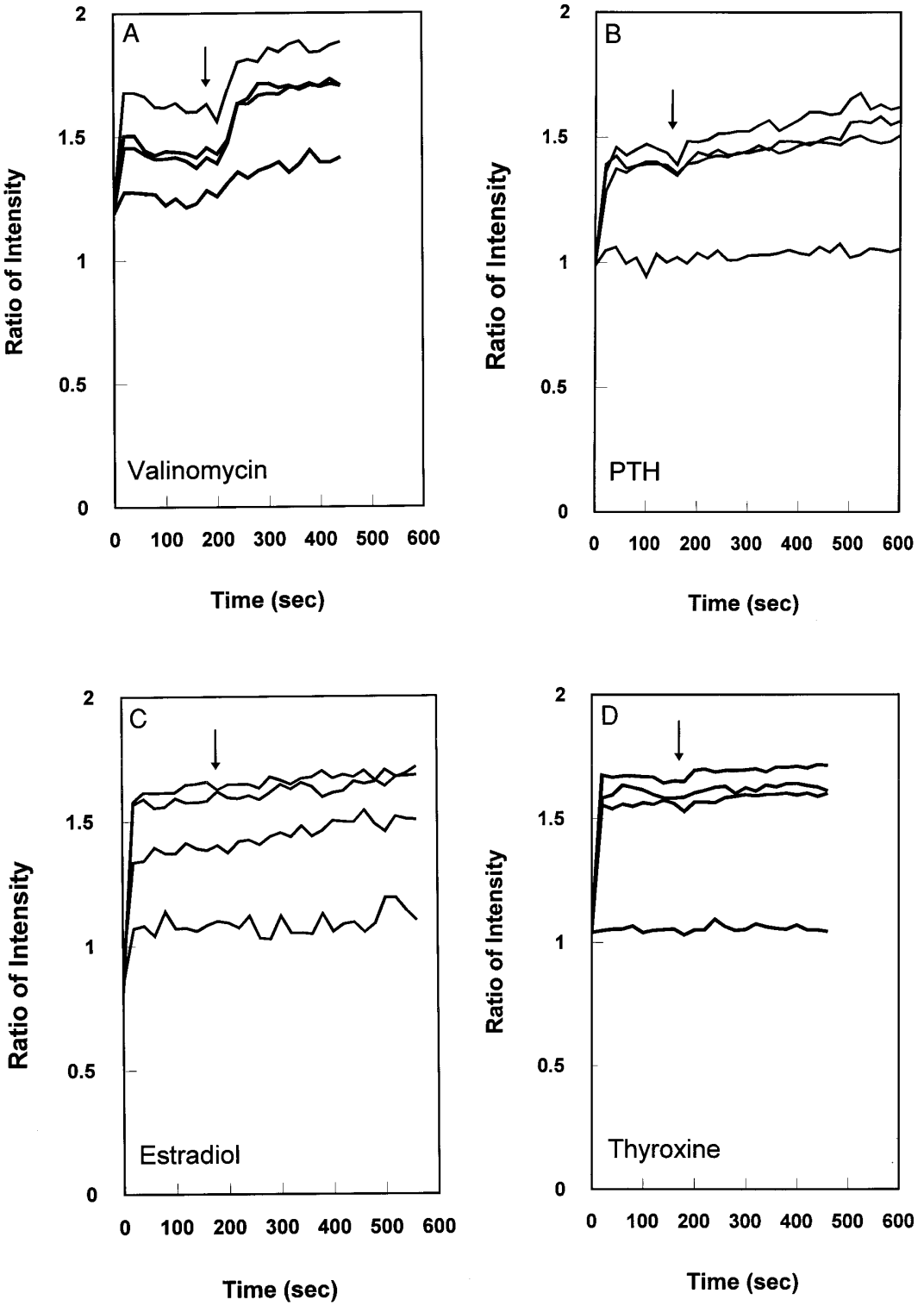

FIG. 2. Changes in the green:red fluorescence ratio of mitochondria in osteoblasts. After establishing a baseline ratio for 200 s, the treatment substance was added (arrows). Each figure shows the response of three individual osteoblasts; the bottom line is the background level of fluorescence immediately outside the cell. For valinomycin (A; 10^{-6} *M*), a rapid shift toward the green suggests that some influx of K^+ reduced mitochondrial membrane potential. Some leakage of the dye occurred following ionophore treatment, as indicated by the slight rise in background fluorescence over time. After 10^{-8} *M* parathyroid hormone treatment (B) or 10^{-8} *M* 17β-estradiol treatment (C), a gradual increase in the ratio was detectable. After 10^{-8} *M* thyroxine treatment (D), no change in the green:red fluorescence ratio was observed.

TABLE 1

Percentage Change in the Green:Red Fluorescence Ratios for Cells Treated with Test Hormones

Treatment[a]	*n*	Average % Change ± SD	*P* ≤
Valinomycin	9	22.54 ± 12.40	0.01
PTH			
200-s treatment	9	6.90 ± 1.90	0.05
300-s treatment		13.90 ± 4.02	0.01
Estrogen			
Overall	15	5.25 ± 4.28	0.5
High response	3	12.53 ± 2.25	0.01
Moderate response	4	5.72 ± 1.30	0.02
Low response	8	2.28 ± 1.26	0.2
Thyroxine			
Overall	12	4.46 ± 2.73	0.5
Moderate response	5	6.86 ± 2.80	0.02
Low response	7	2.83 ± 0.62	0.4
Control	8	3.34 ± 1.40	

Note. Treatment groups are subdivided into high, moderate, or low responsive groups. The column labeled *n* indicates overall number of cells or number of cells in subgroups.

[a] Treatment time of 200 s used, except where noted.

nal that follows PTH–receptor interaction in osteoblasts [14]. The ability of mitochondria to sequester Ca^{2+} when cytosolic calcium rises is a well-known phenomenon [32, 33]. It is now widely held that Ca^{2+} entering mitochondria activates key intramitochondrial enzymes, namely pyruvate dehydrogenase, the 2-oxoglutarate dehydrogenase complex, and NAD-isocitrate dehydrogenase, and thereby causes ATP production even though the proton motive gradient has been momentarily diminished [33]. The uptake of positively charged ions by mitochondria results in decreased mitochondrial membrane potential [33]. In an earlier study, this laboratory has shown that isolated osteoblasts respond to PTH by a rapid and profound efflux of Ca^{2+} through the plasma membrane Ca-ATPase [34]. The major source of the Ca^{2+} was from thapsigargin (IP_3)-sensitive intracellular stores. The phenomenon was maximal at 150 s following PTH administration. During this time frame and immediately subsequent to it mitochondrial uptake of Ca^{2+} could also assist in the rapid restoration of cytosolic Ca^{2+}. The time frame of the hormonal responses of 10–400 s in this study is similar to the time course (10–500 s) of Ca^{2+} efflux from PTH-stimulated osteoblasts [34] and the 0- to 60-s rise of cytosolic Ca^{2+} following estradiol treatment of osteoblast-like cells [21].

Osteoblasts have been shown to respond rapidly to 17β-estradiol through a G-protein-linked plasma membrane receptor [21]. One of the responses to estradiol stimulation reported by Lieberherr and colleagues [21] was a surge in cytosolic Ca^{2+} derived from both IP_3-sensitive cytosolic stores and extracellular fluid. As a consequence, we decided to test the effect of 17β-estradiol on our cells. Red fluorescence was lost in favor of green fluorescence, an indication that mitochondrial membrane potential became reduced. However, only ~50% of the cells responded to estradiol. This may be due to heterogeneity of the cells with respect to stage of differentiation of the 8-day cell cultures wherein not all cells may have developed plasma membrane estrogen receptors; alternatively there could be a rapid internalization of occupied receptors as has been found to occur for PTH and calcitonin receptors in osteoclasts [31, 35]. As is the case for the PTH response, a surge in cytosolic Ca^{2+} may have been restored to basal levels in part through mitochondrial uptake of Ca^{2+} following estradiol treatment. Ca^{2+} is the only second messenger known to enter mitochondria [33] and is, therefore, the likely link between the effects caused by PTH and those caused by estradiol.

Thyroxine was included in this study on the basis of its well-established role in many tissues as a stimulator of basal metabolism and particularly because mitochondrial respiration has been reported to be stimulated by thyroxine in some tissues [36]. In the present study, we found osteoblasts to be unresponsive, suggesting that thyroxine has little or no influence on mitochondrial respiration in osteoblasts.

Studies on neural preparations have shown that membranes do not necessarily have a homogeneous membrane potential throughout; this is believed to be due to an uneven distribution of positively charged ions in cytosol adjacent to plasma membrane [4]. Such a phenomenon is likely to have occurred in the mitochondria examined in the present study since some mitochondria we observed simultaneously possessed regions of green and red fluorescence.

In this study we utilized a fluorescent dye, JC-1, which becomes specifically internalized and concentrated by respiring mitochondria and is consequently useful as a noninvasive tool for detecting rapid changes in mitochondrial activity in living cells. At the same time, this system of analysis is disadvantaged by the problem that external agents acting on cells may mediate their effects on mitochondrial activity indirectly through shifts in cytosolic cations. Since the divalent cation Ca^{2+} is an important intracellular signaling agent, mitochondria in regions of cytosol where levels of Ca^{2+} has surged will likely experience a transient reduction in membrane potential.

The present study has demonstrated that JC-1 can be used to monitor changes in the mitochondrial membrane potential of cultured osteoblasts. Although the signaling pathway has not been revealed, it is likely to be through Ca^{2+} as a second message. Treatment with valinomycin, a potassium ionophore, reduced the membrane potential in all cells tested, as expected. Parathyroid hormone also caused a significant shift toward a

lower mitochondrial membrane potential in all cells tested. Estrogen treatment resulted in a mixed response; in some cells mitochondrial membrane potential did not change but a highly significant change occurred in other cells.

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