J. Membrane Biol. 136, 159–168 (1993)

The Journal of
Membrane
Biology

Characterization of the 1,25-Dihydroxycholecalciferol-stimulated Calcium Influx Pathway in CaCo-2 Cells

Xiao-Ying Tien[1], Christopher Katnik[2], Bahaa M. Qasawa[1], Michael D. Sitrin[1], Deborah J. Nelson[2], Thomas A. Brasitus[1]

[1]Department of Medicine, Section of Gastroenterology, 5841 S. Maryland Avenue, MC 4076, Chicago, Illinois 60637
[2]Department of Neurology, University of Chicago, Chicago, Illinois 60637

Received: 2 March 1992/Revised: 18 June 1993

Abstract. The present studies were conducted to investigate the mechanisms underlying the 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$)-induced increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in individual CaCo-2 cells. In the presence of 2 mM Ca^{2+}, $1,25(OH)_2D_3$-induced a rapid transient rise in $[Ca^{2+}]_i$ in Fura-2-loaded cells in a concentration-dependent manner, which decreased, but did not return to baseline levels. In Ca^{2+}-free buffer, this hormone still induced a transient rise in $[Ca^{2+}]_i$, although of lower magnitude, but $[Ca^{2+}]_i$ then subsequently fell to baseline. In addition, $1,25(OH)_2D_3$ also rapidly induced ^{45}Ca uptake by these cells, indicating that the sustained rise in $[Ca^{2+}]_i$ was due to Ca^{2+} entry. In Mn^{2+}-containing solutions, $1,25(OH)_2D_3$ increased the rate of Mn^{2+} influx which was temporally preceded by an increase in $[Ca^{2+}]_i$. The sustained rise in $[Ca^{2+}]_i$ was inhibited in the presence of external La^{3+} (0.5 mM). $1,25(OH)_2D_3$ did not increase Ba^{2+} entry into the cells. Moreover, neither high external K^+ (75 mM), nor the addition of Bay K 8644 (1 μM), an L-type, voltage-dependent Ca^{2+} channel agonist, alone or in combination, were found to increase $[Ca^{2+}]_i$. $1,25(OH)_2D_3$ did, however, increase intracellular Na^+ in the absence, but not in the presence of 2 mM Ca^{2+}, as assessed by the sodium-sensitive dye, sodium-binding benzofuran isophthalate. These data, therefore, indicate that CaCo-2 cells do not express L-type, voltage-dependent Ca^{2+} channels. $1,25(OH)_2D_3$ does appear to activate a La^{3+}-inhibitable, cation influx pathway in CaCo-2 cells.

Key words: Microfluorimetry—Fura-2—Intracellular Ca^{2+}—Intracellular Na^+—Ca^{2+} channels

Correspondence to: T.A. Brasitus

Introduction

It is well established that 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), the major biologically active metabolite of vitamin D_3, plays an important role in regulating calcium homeostasis and mineral metabolism [3, 27, 35]. The classical target organs for $1,25(OH)_2D_3$ are intestine, bone and kidney [3], but in recent years its receptor has been identified in many other tissues, raising the distinct possibility of other novel functions for this secosteroid [3, 27, 35].

Until recently, it was also well accepted that all of the biological actions of $1,25(OH)_2D_3$ were mediated through a hormone-nuclear/cytosolic vitamin D receptor complex interacting to regulate gene expression in a manner analogous to the mechanisms of action of classical steroid hormones, such as glucocorticoids and estrogens [27]. Over the past several years, however, considerable evidence has accumulated which suggests that $1,25(OH)_2D_3$ may act, at least in part, by mechanisms other than via this general scheme involving steroid hormone-like receptors, gene activation and synthesis of new proteins [4, 5, 7, 10, 21, 22, 24, 34], perhaps directly at the cell surface level.

Our laboratory has recently demonstrated that the addition of $1,25(OH)_2D_3$, in a concentration-dependent manner, rapidly (sec to min) stimulated membrane phosphoinositide (PI) turnover, generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) in suspensions of rat colonocytes [41] and in CaCo-2 cells [40], a cell line derived from a human adenocarcinoma [31]. Moreover, $1,25(OH)_2D_3$ rapidly induced the translocation of protein kinase C (PKC) from the cytosolic to mem-

brane fractions and increased intracellular calcium ($[Ca^{2+}]_i$) in these cells [40, 41].

While the previous population studies suggested that $1,25(OH)_2D_3$ may have increased $[Ca^{2+}]_i$ in these cells via release of calcium from intracellular stores as well as by stimulation of calcium influx pathway(s) [40], the exact mechanisms involved and the sequence of events underlying this phenomenon remained unclear. In the present studies, therefore, it was of interest to determine the activation time course and ionic selectivity of the $1,25(OH)_2D_3$-induced Ca^{2+} influx in individual CaCo-2 cells utilizing micro-fluorimetric techniques.

Materials and Methods

Materials

$1,25(OH)_2D_3$, $24,25(OH)_2D_3$ and $25(OH)D_3$ were kindly provided by Dr. M.R. Uskokovic (Hoffmann-La Roche, Nutley, NJ). All vitamin D_3 metabolites were stored in the dark in absolute ethanol at −20°C. Purity of the metabolites was routinely checked by observing the absorption ratio wavelengths of 264/228 nm. Fura-2 acetoxymethyl ester (Fura-2/AM), sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI/AM) and pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Ionomycin, lanthanum chloride, barium chloride and collagen IV were obtained from Sigma (St. Louis, MO). Ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) was purchased from Fluka (Ronkonkoma, NY). Calcium-45 (^{45}Ca) [2.25 mCi/ml] was obtained from Amersham (Arlington Heights, IL).

Cell Culture and Maintenance

CaCo-2 cells, a human colonic carcinoma cell line originally cloned by Dr. A. Zweibaum [31], were grown as previously described. In brief, the cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) containing 25 mM glucose, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 μg/ml gentamicin, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) and 20% fetal bovine serum (Hyclone, Logan, Utah). The medium was also supplemented with 1% nonessential amino acids and changed every other day.

CaCo-2 cells used in the experiments were seeded onto glass-bottom culture dishes one or two days prior to experiments. The glass-bottom dishes were made as follows: an 18 mm diameter hole was punched into the bottom of a 35 mm plastic culture dish. A 22 × 22 mm acid-cleaned coverslip was then fixed to the outside surface of the dish with Sylgard 184 silicone elastomer to seal the hole. The dishes were placed in an oven at 55°C for 2 hr and sterilized with ethanol and UV light prior to use. All the dishes used for fluorescence studies were precoated with a thin layer of type IV collagen (Sigma) to enhance cell attachment.

Cell Loading with Fluorescence Dyes

The Ca^{2+}-sensitive fluorescence dye, Fura-2 acetoxymethyl ester (Fura-2/AM), was dissolved in 100% dimethyl sulfoxide (DMSO) at 1 mM final concentration. CaCo-2 cells were loaded with 5 μM (final concentration) Fura-2/AM for 1–2 hr at room temperature in serum-free RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD). The loading solution also contained 0.02% pluronic F-127 to obtain maximum dye dispersion and 0.5% bovine serum albumin to prevent nonspecific dye binding. After loading, cells were washed and incubated for 30 min to allow for maximum dye deesterification with an extracellular bath solution consisting of (mM): 145 NaCl, 5.0 KCl, 5.0 $MgCl_2$, 10 HEPES, 10 glucose, 2 $CaCl_2$ at pH 7.2. When a Ca^{2+}-free solution was used, 2 mM $CaCl_2$ was replaced by 1 mM EGTA.

For studies involving the measurement of intracellular Na^+ ($[Na^+]_i$) concentration, CaCo-2 cells were loaded with the Na^+-sensitive fluorescence indicator, sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI/AM in DMSO), according to Harootunian et al. [15]. In brief, the cells were loaded with 10 μM (final concentration) SBFI/AM as described for Fura-2/AM for two to three hours at room temperature.

Microfluorimetry

For the microfluorimetry experiments, a dual wavelength illumination system from Photon Technology International (PTI) [Princeton, NJ] D101 was used which consisted of a 75 W Xenon arc lamp, a variable speed reflective optical chopper under computer control and two monochromators. The system was coupled to the microscope via a fiber optic cable. Excitation light was deflected with a 450 nm dichroic mirror through a 40× phase (Nikon Fluor, N.A. 0.85) objective lens. Emitted fluorescence at 510 nm was collected through an aperture adjusted to the size of the cell by a Hammamatsu R928 photomultiplier tube and photon-counting photometer. In all experiments, a perfusion system (using gravity) at a rate of 2 ml/min was used. Experiments were performed at 35 ± 2°C using a temperature-controlled stage (Brook Ind., Lake Villa, IL).

Cells were chosen for microfluorimetric measurements based on the level of dye loading, i.e., cells loaded to levels at least 10 times higher than background levels were used for these experiments. Changes in $[Ca^{2+}]_i$ or $[Na^+]_i$ following stimulation were expressed as the ratio of 340/380 nm fluorescence intensities. Cells with resting 340/380 nm ratios which were either abnormally low, due to dye compartmentalization or vesicular extrusion during the loading process, or high, presumably due to plasma membrane damage, were excluded from these studies. In general, these latter cells represented less than 5% of the total cell population.

Fluorescence Ratio Imaging

Cells were illuminated using the dual-wavelength illumination, xenon-light source as described above. In the imaging experiments a 20× (Fluor Nikon, N.A. 0.75) objective was used to gather fluorescence data from a number of cells in the field of view. Fluorescence emission was collected with a Hammamatsu c2400 SIT video camera connected to a PTI image processor. Individual frames were summed (number of frames summed depended on loading intensity) and averaged. Frame averages acquired at each wavelength were collected at predetermined intervals and stored on hard disk for off-line analysis. Frame averages at each wavelength were divided pixel by pixel to yield ratio images.

Dye Calibration

For the majority of the experiments, changes in $[Ca^{2+}]_i$ were expressed only as the ratio (R) of dye fluorescence at 340 nm and 380 nm excitation. In those experiments which necessitated an estimate of $[Ca^{2+}]_i$, values for R were converted to Ca^{2+} concentration according to Grynkiewicz et al. [13]:

$$Ca^{2+} = K_D (R - R_{min})/(R_{max} - R)(S_{f2}/S_{b2})$$

where, K_D = 254 nM, R_{min} = 0.28, R_{max} = 5.43, and S_{f2}/S_{b2} = 8.4 (λ_1 = 340 nm and λ_2 = 380 nm) as determined from standard solutions of known Ca^{2+} concentration.

^{45}Ca Uptake into CaCo-2 Cells

^{45}Ca uptake into CaCo-2 cells was measured according to Caffey and Farach [8] with minor modifications. In brief, ^{45}Ca uptake was performed in CaCo-2 cells grown to approximately 60% confluence in six-well plates. Cells were washed two times and incubated at 37°C for 30 min with buffer containing (mM): 145 NaCl, 5.0 KCl, 5.0 $MgCl_2$, 10 HEPES, 10 glucose and 1 $CaCl_2$, pH 7.2. Cells were then incubated with 1 ml of this buffer containing 12 μCi/ml ^{45}Ca in the presence or absence of 10 nM $1,25(OH)_2D_3$ for 1 min at 37°C. The uptake of ^{45}Ca was terminated by washing cells with the ice-cold buffer five times. Cells were subsequently dissolved in 1 ml of 0.5 N NaOH and counted in 10 ml of scintillation cocktail, Budget-Solve (RPI, Mount Prospect, IL). The protein concentrations of these samples were determined according to Bradford [6]. Data are expressed as mean ± SE cpm/mg protein/min.

Statistical Analysis

All results are expressed as means ± SE of n determinations. The paired or unpaired Student's t-test was used for the data analysis as appropriate. P values of 0.05 or less were considered statistically significant.

Results

Changes in $[Ca^{2+}]_i$ In Response to Vitamin D_3 Metabolites in Individual CaCo-2 Cells

The average resting 340/380 nm fluorescence ratio level in Fura-2-loaded CaCo-2 cells was 0.68 ± 0.02 (n = 89, $[Ca^{2+}]_i$ = 51 nM). The addition of $1,25(OH)_2D_3$ (30 nM) to the cells in the presence of 2 mM extracellular Ca^{2+} caused a rapid transient increase in $[Ca^{2+}]_i$ (average ratio changes of 1.36 ± 0.14 above basal level, $[Ca^{2+}]_i$ = 316 nM, n = 31), which then decreased, but did not return to basal levels (0.20 ± 0.03 above basal ratio level, $[Ca^{2+}]_i$ = 97 nM, n = 8) [Fig. 1*A*,*C*]. When the cells were bathed in Ca^{2+}-free solution (< 1 min), $1,25(OH)_2D_3$ (30 nM) caused a rapid transient rise in $[Ca^{2+}]_i$, but of lower magnitude (0.72 ± 0.10 above basal ratio level, $[Ca^{2+}]_i$ = 169 nM, n = 5) than that seen with 2 mM external Ca^{2+}, which then declined to baseline levels (Fig. 1*B*,*C*). The peak transient and sustained increases in $[Ca^{2+}]_i$ induced by $1,25(OH)_2D_3$ were also found to be dependent upon the concentration of secosteroid used (Fig. 2). Taken together, these results suggest that the transient rise in $[Ca^{2+}]_i$ induced by $1,25(OH)_2D_3$ may, at least in part, be secondary to release of this divalent cation from intracellular stores, whereas the sustained rise requires influx of extracellular Ca^{2+}.

Two other naturally occurring vitamin D metabolites, $25(OH)D_3$ (90 nM, n = 8) and $24,25(OH)_2D_3$ (80 nM, n = 5), failed to induce a significant increase in $[Ca^{2+}]_i$ in the presence of 2 mM external Ca^{2+} (*data not shown*).

Effect of $1,25(OH)_2D_3$ on the Uptake of ^{45}Ca into CaCo-2 Cells

To further support our contention that $1,25(OH)_2D_3$ increases $[Ca^{2+}]_i$, at least in part, by inducing the influx of extracellular Ca^{2+} into the cells, assays of ^{45}Ca uptake into CaCo-2 cells by $1,25(OH)_2D_3$ were performed. As seen in Fig. 3, $1,25(OH)_2D_3$ induced a rapid and significant ($P < 0.005$) increase (~2-fold) in ^{45}Ca uptake into the cells over the control levels. These results, therefore, indicate that the sustained rise in $[Ca^{2+}]_i$ was, indeed, due, at least in part, to extracellular Ca^{2+} entry into these cells.

Effects of Mn^{2+} and La^{3+} on $1,25(OH)_2D_3$-Induced Increases in $[Ca^{2+}]_i$

As noted earlier, $1,25(OH)_2D_3$ appeared to increase $[Ca^{2+}]_i$ in CaCo-2 cells by releasing Ca^{2+} from intracellular stores as well as via the activation of a Ca^{2+} influx pathway(s). Based on our earlier studies in these cells [40], it would appear reasonable to suggest that $1,25(OH)_2D_3$ stimulated the release of Ca^{2+} from intracellular stores by increasing IP_3 formation, presumably via the activation of phosphoinositol-phospholipase C (PI-PLC). Since this latter enzyme is, however, Ca^{2+} sensitive, it was theoretically possible that $1,25(OH)_2D_3$ might initially activate a calcium influx pathway, thereby, activating PI-PLC which, in turn, led to an increase in IP_3 and a subsequent further rise in $[Ca^{2+}]_i$. To examine the sequence of events leading to Ca^{2+} influx secondary to $1,25(OH)_2D_3$ stimulation, we performed experi-

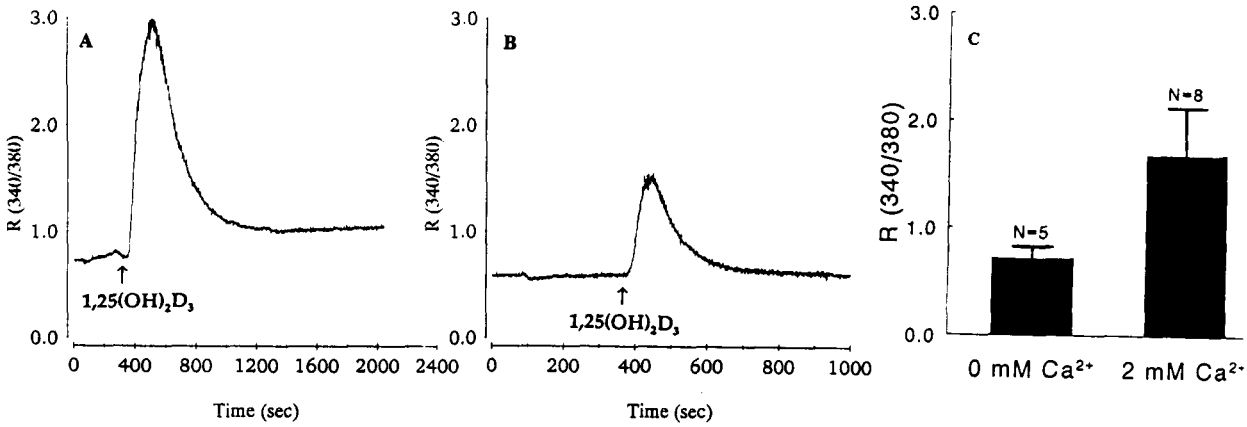

Fig. 1. The effect of external Ca^{2+} on the $1,25(OH)_2D_3$-induced increases in $[Ca^{2+}]_i$ in individual Fura-2-loaded CaCo-2 cells. Traces represent changes in the ratio of Fura-2 dye emission at 510 nm following dye excitation at 340 and 380 nm following exposure of single cells to $1,25(OH)_2D_3$. Increases in $[Ca^{2+}]_i$ following exposure of the cells to $1,25(OH)_2D_3$ (30 nM) were expressed as increases in the ratio (R) of dye fluorescence 340 and 380 nm. (A) In a 2 mM Ca^{2+} buffer, $1,25(OH)_2D_3$ caused a rapid increase in $[Ca^{2+}]_i$, which subsequently decreased, but remained above the baseline level. (B) Change in $[Ca^{2+}]_i$ in a cell exposed to Ca^{2+}-free buffer less than 1 min prior to stimulation with $1,25(OH)_2D_3$. Note that under Ca^{2+}-free conditions $1,25(OH)_2D_3$-induced a smaller transient rise in $[Ca^{2+}]_i$ which decreased to initial levels. (C) Dependence of the amplitude of the peak of the $1,25(OH)_2D_3$-induced transient increase in $[Ca^{2+}]_i$ on external Ca^{2+}. In the absence of external Ca^{2+}, the mean peak amplitude of the transient response was less than that observed in 2 mM Ca^{2+} ($P < 0.05$). N represents the number of individual cells in response to $1,25(OH)_2D_3$ stimulation from 5–8 separate experiments.

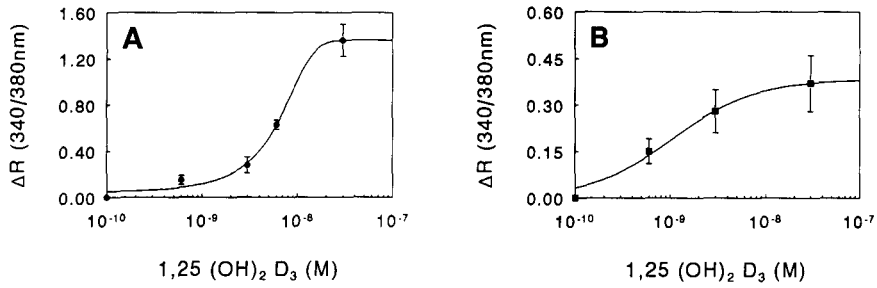

Fig. 2. Concentration dependence of the amplitude of the peak and sustained components of the $1,25(OH)_2D_3$-induced increase in $[Ca^{2+}]_i$ in the presence of 2 mM extracellular Ca^{2+} in Fura-2-loaded CaCo-2 cells. (A) Amplitude of the peak of the transient response as a function of the concentration of $1,25(OH)_2D_3$. (B) Amplitude of the sustained portion of the response taken at 500 sec after the peak response. Each data point represents the mean ± SE from at least five different individual cells obtained in separate microfluorimetric experiments.

ments in Fura-2-loaded CaCo-2 cells exposed to an external solution containing both 0.5 mM Mn^{2+} and 2 mM Ca^{2+}. Emission at 510 nm was monitored following dye excitation at 357 nm (Ca^{2+}-Fura-2 isosbestic point) and 340 nm. Changes in fluorescence at the isosbestic point (357 nm) reflect quenching due to Mn^{2+} influx and are not influenced by changes in $[Ca^{2+}]_i$, whereas increases in the fluorescence intensity at the Ca^{2+}-sensitive wavelength (340 nm), indicate a rise in $[Ca^{2+}]_i$ due to release from internal stores [26].

Figure 4 shows representative tracings from a Mn^{2+} quench experiment in the presence of 2 mM external Ca^{2+}. Similar results were obtained in a total of seven cells. There was a gradual decrease in fluorescence intensity when cells were exposed to a solution containing 0.5 mM Mn^{2+}, indicating slow entry of Mn^{2+} into the unstimulated cells. The rate of Mn^{2+} quenching, as assessed by the rate of fall of fluorescence intensity (Fig. 4), however, was increased approximately 2.5 ± 0.5-fold ($n = 7$) after addition of $1,25(OH)_2D_3$ (30 nM). Moreover, the rapid rise in $[Ca^{2+}]_i$, as noted at 340 nm wavelength, occurred approximately 20 sec prior to this increased rate of quenching by Mn^{2+}. These data indicate that $1,25(OH)_2D_3$ causes a rise in $[Ca^{2+}]_i$ prior to activation (opening) of a cation influx pathway. This sequence of events, in conjunction with our

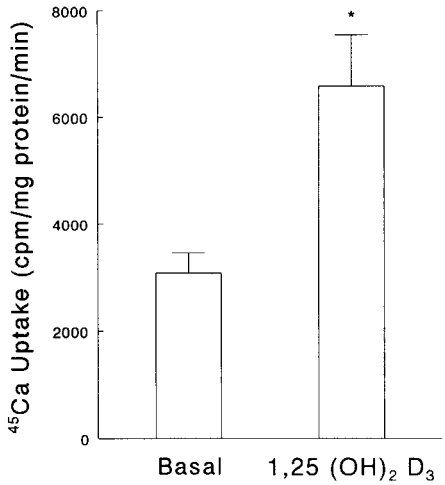

Fig. 3. The effect of $1,25(OH)_2D_3$ on ^{45}Ca uptake into CaCo-2 cells. ^{45}Ca uptake was assayed for 1 min with or without $1,25(OH)_2D_3$ (10 nM). Data are expressed as mean ± SE of 8–9 separate determinations. *Value significantly different from the basal, $P < 0.005$.

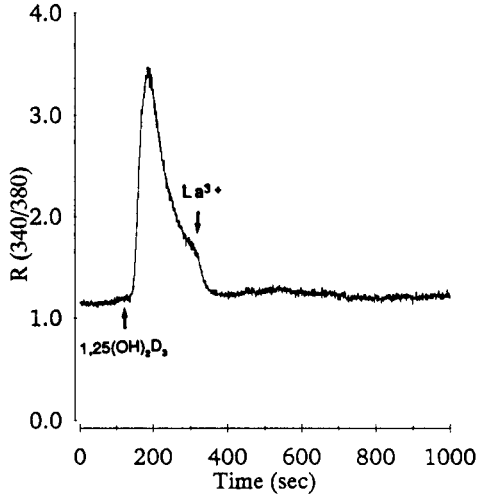

Fig. 5. The effect of extracellular La^{3+} on the sustained increase in $[Ca^{2+}]_i$ induced by $1,25(OH)_2D_3$ (30 nM). Addition of La^{3+} (0.5 mM) caused a reduction of $[Ca^{2+}]_i$ during the sustained phase of the response to the baseline level, presumably through inhibition of a La^{3+}-sensitive Ca^{2+} influx pathway.

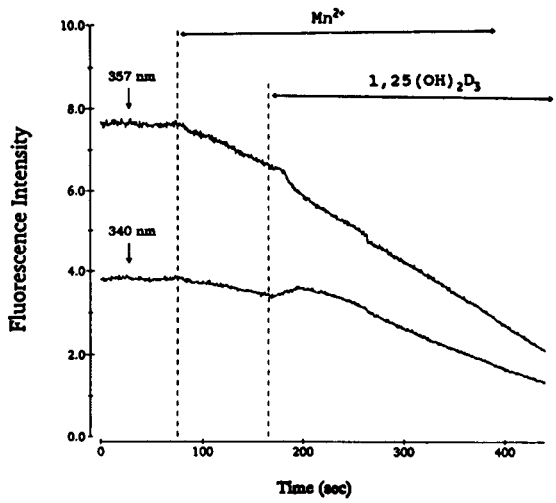

Fig. 4. The effect of $1,25(OH)_2D_3$ on the rate of Mn^{2+} influx into a single CaCo-2 cell. Changes in Fura-2 fluorescence were measured following excitation at 340 nm and 357 nm (Ca^{2+}-Fura-2 isosbestic point) before and after exposure of the cell to $1,25(OH)_2D_3$ in the presence of 2 mM external Ca^{2+}. Similar data were obtained in separate experiments performed on a total of seven cells. Fluorescence intensity is expressed as counts/sec × 10^5. In the absence of $1,25(OH)_2D_3$, Mn^{2+} entered CaCo-2 cells slowly seen as a gradual decrease in fluorescence intensity at 357 nm (fluorescence quenching). Addition of $1,25(OH)_2D_3$ (30 nM) induced a rapid increase in fluorescence intensity at the Ca^{2+}-sensitive wavelength (340 nm) signaling internal Ca^{2+} release, which occurred approximately 20 sec prior to an increase in the rate of Mn^{2+} influx, detectable as an increase in fluorescence quenching at 357 nm.

previous studies which demonstrated that $1,25(OH)_2D_3$ stimulated PI breakdown [40], strongly indicate that the initial rise in $[Ca^{2+}]_i$ is due to IP_3-induced release of intracellular stores of Ca^{2+} followed by influx of extracellular Ca^{2+}.

Previous studies have shown that La^{3+} inhibits receptor-mediated Ca^{2+} influx [28]. It was, therefore, of interest to determine whether La^{3+} influenced the sustained increase in $[Ca^{2+}]_i$ induced by $1,25(OH)_2D_3$. As shown in Fig. 5, the addition of La^{3+} during this phase promptly reduced $[Ca^{2+}]_i$ to baseline levels ($n = 6$). In certain studies [29], La^{3+} has also been shown to enter cells. Since La^{3+} has a high affinity for Fura-2 ($K_d \sim 10^{-12}$ M with a high quantum yield) [43], entry into CaCo-2 cells would have been expected to increase the fluorescence ratio rather than decrease it as was observed in the present experiments.

Studies on the Voltage-Sensitivity of Ca^{2+} Influx Pathway in CaCo-2 Cells

Given the limited Mn^{2+} permeability of the $1,25(OH)_2D_3$-stimulated influx pathway, further studies investigating the Ba^{2+} permeability of the entry pathway were performed. Similar experiments have been carried out on receptor-activated Ca^{2+} influx pathways in other preparations [28]. We examined the Ba^{2+} permeability of the $1,25(OH)_2D_3$-activated influx pathway by monitoring fluorescence at the Ca^{2+}-independent, but Ba^{2+}-dependent wavelength (360 nm), as well as at a Ca^{2+}-dependent,

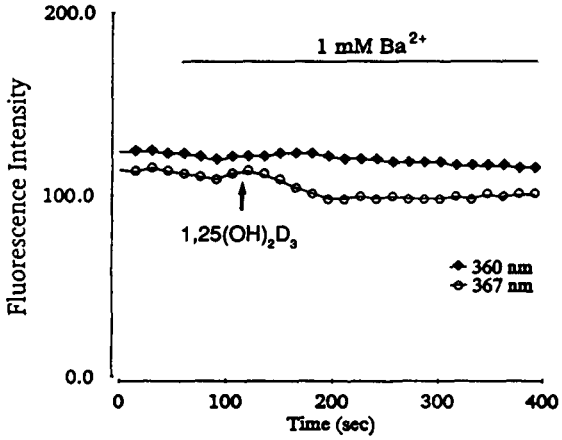

Fig. 6. Ba^{2+} selectivity of the $1,25(OH)_2D_3$-induced Ca^{2+} influx pathway. Fluorescence intensity was measured at 367 nm (Ba^{2+}-insensitive, Ca^{2+}-sensitive wavelength), and 360 nm (Ba^{2+}-sensitive, Ca^{2+}-insensitive wavelength). Ba^{2+} (1 mM) entry was monitored following stimulation by $1,25(OH)_2D_3$ (30 nM) in a fluorescence imaging experiment. Averaged intensity changes for an individual cell in a given field of view was determined over time. Fluorescence intensity is expressed in arbitrary units. The addition of $1,25(OH)_2D_3$ did not increase the fluorescence signal at 360 nm, indicating that Ba^{2+} did not enter the cell; whereas, the $1,25(OH)_2D_3$-induced release of internal Ca^{2+} could be detected as a rapid decrease in fluorescence intensity at the Ca^{2+}-sensitive wavelength (367 nm).

but Ba^{2+}-independent wavelength (367 nm) [28]. An increase in the 360 nm signal, therefore, would indicate an increase in the rate of Ba^{2+} entry, while a decrease in the 367 nm signal (which decreases in intensity with Ca^{2+} binding to the dye) would indicate an increase in $[Ca^{2+}]_i$. As noted in Fig. 6, Ba^{2+} (1 mM) did not increase the 360 nm fluorescence intensity in unstimulated cells ($n = 10$). After addition of $1,25(OH)_2D_3$, the fluorescence intensity at 360 nm did not change ($n = 14$) but the fluorescence intensity at 367 nm (Ca^{2+} sensitive), however, decreased ($n = 14$), indicating that Ba^{2+} did not enter a $1,25(OH)_2D_3$-activated Ca^{2+} influx pathway. It is unlikely that this latter effect was caused by differential affinities of Fura-2 for Ba^{2+} *vs.* Ca^{2+}, since published estimates of these affinities ($K_D \sim 254$ nM for Ca^{2+} and ~780 nM for Ba^{2+} in the presence of Ca^{2+}) are within the same order of magnitude [28].

To further determine whether CaCo-2 cells possess voltage-sensitive Ca^{2+} influx pathways that might be involved in the $1,25(OH)_2D_3$-induced $[Ca^{2+}]_i$ rise, we used a depolarizing concentration of external K^+ (75 mM). As seen in Fig. 7*A*, this concentration of extracellular K^+ did not affect $[Ca^{2+}]_i$ ($n = 6$). The effects of the Ca^{2+} L-type channel agonist, Bay K 8644 (1 μM) alone (Fig. 7*B*) or in combination with high K^+ (75 mM) (Fig. 7*C*) on $[Ca^{2+}]_i$ were also examined. As can be seen in this figure, neither Bay K 8644 alone ($n = 6$), nor in combination with high K^+ ($n = 4$) increased $[Ca^{2+}]_i$.

Effect of $1,25(OH)_2D_3$ on $[Na^+]_i$ in CaCo-2 Cells

To assess the monovalent cation permeability of the $1,25(OH)_2D_3$-stimulated influx pathway(s), CaCo-2 cells were loaded with the sodium-sensitive fluorescence indicator, SBFI/AM, for determination of cytosolic free Na^+ ($[Na^+]_i$). Fluorescence ratio changes of 340/380 nm were used to monitor the response of $[Na^+]_i$ to $1,25(OH)_2D_3$ (30 nM). As shown in Fig. 8, $1,25(OH)_2D_3$-induced a slow increase in $[Na^+]_i$ in a Ca^{2+}-free solution ($n = 4$), whereas it failed to alter $[Na^+]_i$ in the presence of 2 mM external Ca^{2+} ($n = 5$).

Discussion

Recently, utilizing CaCo-2 cell suspensions, our laboratory has reported that the addition of $1,25(OH)_2D_3$, in a concentration-dependent manner, rapidly (sec to min) stimulated membrane phosphoinositide turnover, generating DAG and IP_3 [40]. In these preparations, $1,25(OH)_2D_3$ also rapidly induced the cellular redistribution of PKC from the cytosolic to membrane fractions and increased $[Ca^{2+}]_i$. The mechanisms responsible for the increase of $[Ca^{2+}]_i$ by $1,25(OH)_2D_3$ in these prior experiments were, however, unclear.

The present studies in individual Fura-2-loaded CaCo-2 cells, utilizing microfluorimetry techniques, have confirmed and extended these prior observations by examining the time course of Ca^{2+} influx and the ion selectivity of the influx pathway(s) in response to the addition of $1,25(OH)_2D_3$. In agreement with our previous findings [40], $1,25(OH)_2D_3$ evoked a transient rise in $[Ca^{2+}]_i$, which then decreased, but did not return to baseline levels, i.e., was sustained for at least 1,500 sec.

These rapid effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$, moreover, could not be produced in CaCo-2 cells by the addition of higher concentrations of two other vitamin D_3 metabolites, $25(OH)D_3$ and $24,25(OH)_2D_3$. These observations, therefore, are in general agreement with the specific nature of the actions of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ in several other cell types [24, 38, 39, 41].

The transient rise in $[Ca^{2+}]_i$ due to $1,25(OH)_2D_3$, while reduced in magnitude, was still found to be present in cells exposed to a Ca^{2+}-free buffer. These findings indicate that, at least in part, the $1,25(OH)_2D_3$-induced transient increase in $[Ca^{2+}]_i$ was due to release of Ca^{2+} from intracellular stores, presumably via the generation of IP_3 by this secosteroid [40]. In addition, these data suggest that significant store depletion occurs rapidly in Ca^{2+}-

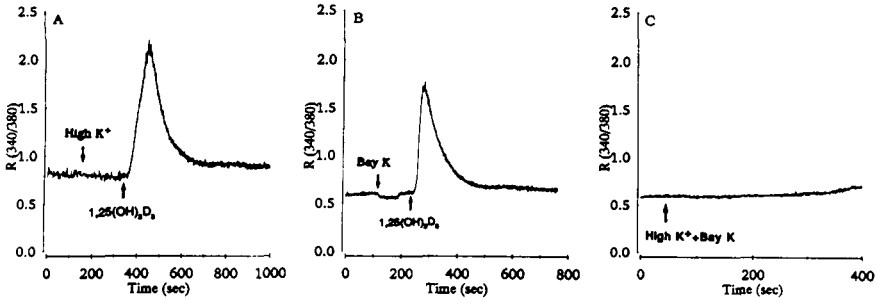

Fig. 7. Voltage insensitivity of the Ca^{2+} influx pathway in CaCo-2 cells. Traces were obtained in separate microfluorimetric experiments on individual cells. $[Ca^{2+}]_i$ did not change in response to: (*A*) high external K^+ (75 mM); (*B*) Bay K 8644 (1 μM) and (*C*) combination of high K^+ (75 mM) with Bay K 8644 (1 μM). These same cells, however, were responsive to $1,25(OH)_2D_3$ (30 nM) (*A* and *B*). Cells were exposed to $1,25(OH)_2D_3$ in high K^+ and Bay K 8644 containing solutions. It should be noted that Bay K 8644 appeared to inhibit the sustained plateau phase of the $[Ca^{2+}]_i$ transient, indicating that it may inhibit Ca^{2+} influx.

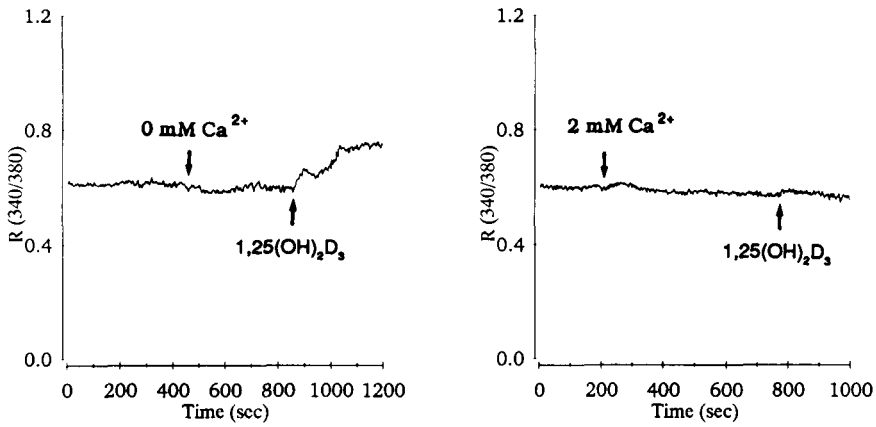

Fig. 8. Na^+ influx after exposure to $1,25(OH)_2D_3$ in the presence or absence of external Ca^{2+} as determined in individual SBFI/AM-loaded CaCo-2 cells. $[Na^+]_i$ increased after the addition of $1,25(OH)_2D_3$ (30 nM), as detected by an increase in the 340/380 nm ratio when cells were bathed in a Ca^{2+}-free solution (left panel). Stimulation with $1,25(OH)_2D_3$, however, failed to alter $[Na^+]_i$ in the presence of 2 mM external Ca^{2+} (right panel).

free buffer. It should also be noted that this initial rise in $[Ca^{2+}]_i$ induced by $1,25(OH)_2D_3$ temporally preceded the accelerated Mn^{2+} quenching of Fura-2 fluorescence in response to the secosteroid; again supporting the contention that the transient rise in $[Ca^{2+}]_i$ was derived from the release of this divalent cation from intracellular stores rather than via a Ca^{2+} influx pathway(s).

Alternatively, this secosteroid could have theoretically increased $[Ca^{2+}]_i$, via inhibition of Na^+/Ca^{2+} exchange or via inhibition of Ca^{2+}-ATPase. The former possibility, however, was ruled out by previous experiments in which the replacement of extracellular Na^+ by Tris or N-methyl-glucamine gluconate failed to alter the effects of $1,25(OH)_2D_3$ on $[Ca^{2+}]_i$ in these cells [40]. While not directly addressed in the present experiments, the latter possibility also appears unlikely in view of prior studies which have shown that $1,25(OH)_2D_3$ stimulates intestinal Ca^{2+}-ATPase [44]. Taken together, these observations strongly indicate, therefore, that the sustained rise in $[Ca^{2+}]_i$ by $1,25(OH)_2D_3$ was not due to an inhibition of Ca^{2+} efflux, but rather to a stimulation of Ca^{2+} influx across the plasma membranes of CaCo-2 cells. Moreover, in support of this contention, $1,25(OH)_2D_3$ induced the rapid uptake of ^{45}Ca by CaCo-2 cells and La^{3+} promptly reduced the sustained rise in $[Ca^{2+}]_i$ induced by $1,25(OH)_2D_3$ to baseline levels.

In the past, epithelial cells had not been thought to possess voltage-sensitive Ca^{2+} influx pathways [37]. More recently, however, a number of studies in various epithelial cells, including rabbit ileal enterocytes [16] and chick duodenal cells [11], have suggested that these cells may possess such Ca^{2+} influx pathways. In the present experiments, however, neither depolarization of CaCo-2 cells by high extracellular K^+, nor the addition of the voltage-sensitive, L-type Ca^{2+} channel agonist, Bay K 8644, alone or in combination with high extracellular K^+ was found to increase $[Ca^{2+}]_i$. Moreover, Ba^{2+} which classically has been found to enter cells via

voltage-sensitive Ca^{2+} channels [28], also failed to permeate the $1,25(OH)_2D_3$-activated cation influx pathway in CaCo-2 cells. These results strongly argue against the presence of L-type voltage-sensitive Ca^{2+} channels in CaCo-2 cells. While the present studies do not exclude the existence of T-type voltage-sensitive Ca^{2+} channels in these cells, we believe that the possibility is unlikely since such Ca^{2+} channels, to our knowledge, have not been reported to date in polarized epithelial cells.

Additionally, it should be noted that several properties of this $1,25(OH)_2D_3$-activated cation influx pathway, including its inhibition by La^{3+}, its permeability to Mn^{2+}, its impermeability to Ba^{2+} and its voltage independence, are similar to those described for receptor-operated, voltage-independent Ca^{2+} channels (ROCCs) [28]. Such channels, which have also been termed second messenger-operated channels, have been described in a number of different cell types [25, 33, 36], including rat hepatocytes [20]. The mechanism(s) by which agonist-receptor interaction stimulates Ca^{2+} influx remains unclear. Previous studies, however, have suggested that ROCCs may be regulated by increases in IP_3 alone [19, 30] or in combination with inositol 1,3,4,5-tetrakisphosphate [9, 23], as well as via alterations in $[Ca^{2+}]_i$ [32], and guanine nucleotide binding proteins [17, 18]. In this regard, it is of interest to note that the present results are consistent with the "capacitative" model for Ca^{2+} influx in nonexcitable cells proposed by Putney [33].

The present experiments using the Na^+-sensitive dye, SBFI/AM, demonstrated that $1,25(OH)_2D_3$ also increased Na^+ influx and raised $[Na^+]_i$, but only in cells bathed in Ca^{2+}-free buffer. Similar results have been obtained in studies of voltage-dependent calcium channels [1]. Thus, the non-voltage-dependent cation channels described in the present study in CaCo-2 cells may share this property with voltage-dependent cation channels.

While several investigators have previously postulated that a receptor for $1,25(OH)_2D_3$ may be present on cell surface membranes [2, 22] which could be linked to a ROCC, to date, the existence of such a putative receptor has not been established. Recently, however, our laboratory, utilizing CaCo-2 cells grown as tight polarized monolayers, has found that $1,25(OH)_2D_3$ activates the phosphoinositol signal transduction cascade, specifically in the basolateral membrane region of these cells [42]. These findings, therefore, would strongly suggest that if a receptor for $1,25(OH)_2D_3$ does exist on the surface of CaCo-2 cells, it is localized on the basolateral plasma membrane. The existence of such a receptor for $1,25(OH)_2D_3$ and its possible relationship to the $1,25(OH)_2D_3$-activated cation influx pathway in CaCo-2 cells is obviously unclear at this time and will require additional studies.

In other cell types, increases in cytosolic calcium due to agonist-stimulated release of intracellular stores and/or Ca^{2+} influx appear to be important in the regulation of a number of different cellular functions, including growth and differentiation [12]. The sustained rise in $[Ca^{2+}]_i$ secondary to the activation of this influx pathway by $1,25(OH)_2D_3$ may also have important physiological consequences in CaCo-2 cells. In this regard, prior studies from our laboratory have recently demonstrated that the sustained rise in Ca^{2+} secondary to this secosteroid inhibited Na^+/H^+ exchange in these cells via a Ca^{2+}/calmodulin-dependent process [39]. Since alterations in Na^+/H^+ exchange appear to play a critical role in the regulation of cellular proliferation [12] and $1,25(OH)_2D_3$ has been shown by our laboratory to also decrease the proliferation rates of CaCo-2 cells [14], it is entirely possible that these actions of $1,25(OH)_2D_3$ are related. Further studies will be necessary, however, to clarify whether activation of the presently described cation influx pathway by $1,25(OH)_2D_3$ is linked to the hormone's antiproliferative effects in CaCo-2 cells.

The authors acknowledge the expert secretarial assistance of Lynn Nelson. This research was supported in part by the following grants: 5P30DK26687 (Clinical Nutrition Research Unit), P30DK42086 (Digestive Diseases Research Core Center), DK39573 and CA36745 from the U.S. Public Health Science (TAB) and DK44840 and GM36823 from the U.S. Public Health Service (DJN). Thomas A. Brasitus is the recipient of a MERIT Award from the National Cancer Institute.

References

1. Almers, W., McCleskey, E.W. 1983. Non-selective conductance in calcium channels of frog muscles. Calcium selectively in a single-file pore. *J. Physiol.* **353:**585–608
2. Barsony, J., Marx, S.J. 1988. Receptor-mediated rapid action of 1α,25-dihydroxycholecalciferol: Increase of intracellular cGMP in human skin fibroblast. *Proc. Natl. Acad. Sci. USA* **85:**1223–1226
3. Bell, N.H. 1985. Vitamin D-endocrine system. *J. Clin. Invest.* **76:**1–6
4. Ben Nasr, L., Monet, J.D., Lucas, P.A. 1988. Rapid (10-minute) stimulation of rat duodenal alkaline phosphatase activity by 1,25-dihydroxyvitamin D_3. *Endocrinology* **123:** 1778–1782
5. Bordeau, A., Atmani, F., Grosse, B., Lieberherr, M. 1990. Rapid effects of 1,25-dihydroxyvitamin D_3 and extracellular Ca^{2+} on phospholipid metabolism in dispersed porcine parathyroid cells. *Endocrinology* **127:**2739–2743
6. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254
7. Brasitus, T.A., Dudeja, P.K., Eby, B., Lau, K. 1986. Correc-

tion by 1,25-dihydroxycholecalciferol of the abnormal fluidity and lipid composition of enterocyte brush border membranes in vitamin D deprived rats. *J. Biol. Chem.* **261:**16404–16409
8. Caffrey, J.M., Farach, M.C. 1988. A monoclonal antibody specifically modulates dihydropyridine-sensitive calcium current in BC_3H_1 myocytes. *Mol. Pharmacol.* **34:**518–526
9. Changya, L., Gallacher, D.V., Irvine, R.F., Potter, B.V.L., Petersen, O.H. 1989. Inositol 1,3,4,5-tetrakisphosphate is essential for sustained activation of the Ca^{2+}-dependent K^+ current in single internally perfused mouse lacrimal acinar cells. *J. Membrane Biol.* **109:**85–93
10. De Boland, A.R., Nemere, I., Norman, A.W. 1990. Ca^{2+}-channel agonist Bay K8644 mimics $1,25(OH)_2$-vitamin D_3 rapid enhancement of Ca^{2+} transport in chick perfused duodenum. *Biochem. Biophys. Res. Commun.* **166:**217–222
11. De Boland, A.R., Norman, A.W. 1990. Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D_3-dependent transcaltachia (the rapid stimulation of duodenal Ca^{2+} transport). *Endocrinology* **127(5):**2475–2480
12. Grinstein, S., Rotin, D., Mason, M.J. 1989. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* **988:**73–97
13. Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:**3440–3450
14. Halline, A.G., Davidson, N.O., Sitrin, M.D., Tietze, C., Alpers, D.H., Brasitus, T.A. 1990. Effects of $1,25(OH)_2$ vitamin D_3 on proliferation and differentiation of CaCo-2 cells. *Gastroenterology* **98(5):**A414
15. Harootunian, A.T., Kao, J.P.Y., Eckert, B.K., Tsien, R.Y. 1989. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *J. Biol. Chem.* **264(32):**19458–19467
16. Homaidan, F.R., Donowitz, M., Weiland, G.A., Sharp, G.W.G. 1989. Two calcium channels in basolateral membranes of rabbit ileal epithelial cells. *Am. J. Physiol.* **257:**G86–G93
17. Hughes, B.P., Barritt, G.J. 1987. The stimulation by sodium fluoride of plasma-membrane Ca^{2+} inflow in isolated hepatocytes: Evidence that a GTP-binding regulatory protein is involved in the hormonal stimulation of Ca^{2+} inflow. *Biochem. J.* **245:**41–47
18. Hughes, B.P., Crofts, J.N., Auld, A.M., Read, L.C., Barritt, G.J. 1987. Evidence that a pertussis-toxin-sensitive substrate is involved in the stimulation by epidermal growth factor and vasopressin of plasma-membrane Ca^{2+} inflow in hepatocytes. *Biochem. J.* **248:**911–918
19. Irvine, R.F. 1990. 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates—a possible mechanism. *FEBS Lett.* **263(1,5–9):**5–9
20. Kass, G.E.N., Llopis, J., Chow, S.C., Duddy, S.K., Orrenius, S. 1990. Receptor-operated calcium influx in rat hepatocytes. *J. Biol. Chem.* **265(29):**17486–17492
21. Kurnik, B.R.C., Huskey, M., Hagerty, D., Hruska, K.A. 1986. Vitamin D metabolites stimulate phosphatidylcholine transfer to renal brush-border membranes. *Biochim. Biophys. Acta* **858:**47–55
22. Lieberherr, M., Grosse, B., Duchambon, P., Drueke, T. 1989. A functional cell surface type receptor is required for the early action of 1,25-dihydroxyvitamin D_3 on the phosphoinositide metabolism in rat enterocytes. *J. Biol. Chem.* **193:**265–275
23. Luckhoff, A., Clapham, D.E. 1992. Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel. *Nature* **355(23):**356–358
24. Maclaughlin, J.A., Cantley, L.C., Holick, M.F. 1990. $1,25(OH)_2D_3$ increases calcium and phosphatidylinositol metabolism in differentiating cultured human keratinocytes. *J. Nutr. Biochem.* **1:**81–87
25. Meldolesi, J., Clementi, E., Fasolato, C., Zacchetti, D., Pozzan, T. 1991. Ca^{2+} influx following receptor activation. *TIPS* **12:**289–292
26. Merritt, J.E., Jacob, R., Hallam, T.J. 1989. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J. Biol. Chem.* **264:**1522–1527
27. Minghetti, P.P., Norman A.W. 1988. $1,25(OH)_2$-vitamin D_3 receptors: gene regulation and genetic circuitry. *FASEB J.* **2:**3043–3053
28. Murray, R., Kotlikoff, M. 1991. Receptor-activated calcium influx in human airway smooth muscle cells. *J. Physiol.* **435:**123–144
29. Peeters, G.A., Kohmoto, O., Barry, W.H. 1989. Detection of La^{3+} influx in ventricular cells by indo-1 fluorescence. *Am. J. Physiol.* **256:**C351–C357
30. Penner, R., Matthews, G., Neher, E. 1988. Regulation of calcium influx by second messengers in rat mast cells. *Nature* **334(11):**499–504
31. Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J, Zweibaum, A. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47:**323–330
32. Poggioli, J., Mauger, J.-P., Guesdon, F., Claret, M. 1985. A regulatory calcium-binding site for calcium channel in isolated rat hepatocytes. *J. Biol. Chem.* **260(6):**3289–3294
33. Putney, J.W., Jr. 1990. Capacitative calcium entry revisited. *Cell Calcium* **11:**611–624
34. Rasmussen, H., Matsumoto. T., Fontaine, O., Goodman, D.B.P. 1982. Role of changes in membrane lipid structure in the action of 1,25-dihydroxyvitamin D_3. *Fed. Proc.* **41:**72–77
35. Reichel, H., Koeffler, H.P., Norman, A.W. 1989. The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.* **320:**980–991
36. Rink, T.J. 1990. Receptor-mediated calcium entry. *FEBS Lett.* **268(2):**381–385
37. Siemer, C., Gogelein, H. 1992. Activation of nonselective cation channels in the basolateral membrane of rat distal colon crypt cells by prostaglandin E_2. *Pfluegers Arch.* **420:**319–328
38. Sugimoto, T., Ritter, C., Slatopolsky, E., Morissey, J. 1988. Effect of 1,25-dihydroxyvitamin D_3 on phospholipid metabolism in cultured bovine parathyroid cells. *Endocrinology* **122:**2387–2392
39. Tornquist, K., Tashjian, A.H., Jr. 1989. Dual actions of 1,25-dihydroxycholecalciferol on intracellular Ca^{2+} in GH_4C_1 cells: Evidence for effects on voltage-operated Ca^{2+} channels and Na^+/Ca^{2+} exchange. *Endocrinology* **124(6):**2765–2776
40. Wali, R.K., Baum, C.L., Bolt, M.J.G., Brasitus, T.A., Sitrin, M.D. 1992. 1,25-dihydroxyvitamin D_3 inhibits Na^+-H^+ exchange by stimulating membrane phosphoinositide turnover and increasing cytosolic calcium in CaCo-2 cells. *Endocrinology* **131(3):**1125–1133
41. Wali, R.K., Baum, C.L., Sitrin, M.D., Brasitus, T.A. 1990. $1,25(OH)_2$ vitamin D_3 stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic

calcium in rat colonic epithelium. *J. Clin. Invest.* **85:**1296–1303

42. Wali, R.K., Bolt, M.J.G., Tien, X.-Y., Brasitus, T.A., Sitrin, M.D. 1992. Differential effect of 1,25 dihydroxycholecal-ciferol on phosphoinositide turnover in the antipodal plasma membranes of colonic epithelial cells. *Biochem. Biophys. Res. Commun.* **187(2):**1128–1134
43. Yamaguchi, D.T., Green, J., Kleeman, C.R., Muallem, S. 1989. Properties of the depolarization-activated calcium and barium entry in osteoblast-like cells. *J. Biol. Chem.* **264(1):**197–204
44. Zelinski, J.M., Sykes, D.E., Weiser, M.M. 1991. The effect of vitamin D on rat intestinal plasma membrane Ca-pump mRNA. *Biochem. Biophys. Res. Commun.* **179(2):**749–755