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Rapid effects of 1α,25(OH)$_2$D3 in resting human peripheral blood mononuclear cells

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Abstract

The steroid hormone 1α,25(OH)$_2$D3 produces biological responses via both genomic and nongenomic mechanisms. Stimulation of rapid, nongenomic responses by 1α,25(OH)$_2$D3 has been postulated to result from interaction of the ligand with cell membrane 1α,25(OH)$_2$D3 receptors and to involve membrane receptors. We examined the rapid effects of 1α,25(OH)$_2$D3 on calcium mobilization and calcium entry into resting human peripheral blood mononuclear cells isolated from healthy volunteers. We also investigated the possible involvement of purinergic receptors in this action. 1α,25(OH)$_2$D3 induced a time-dependent increase in intracellular calcium concentration ($[Ca^{2+}]_i$). The initial 1α,25(OH)$_2$D3-stimulated calcium increment was sensitive to thapsigargin (Tg), indicating its origins in calcium release from intracellular stores. 2-Aminoethyldiphenyl borate (2APB), an inhibitor of capacitative calcium entry, caused a significant $[Ca^{2+}]_i$ decrease in human cells treated with 1α,25(OH)$_2$D3. Furthermore, in contrast to observations in osteoblasts and skeletal muscle cells, nifedipine had no effect on 1α,25(OH)$_2$D3-induced calcium entry, suggesting that L-type calcium channels were not implicated in this action. Besides, 1α,25(OH)$_2$D3 prevented the calcium entry induced by 3′-*O*-(4-benzoyl)benzoyl-adenosine 5′-triphosphate (BzATP), a specific agonist of purinergic P2X$_7$ receptors. This finding was further confirmed by 1α,25(OH)$_2$D3-induced reduction of BzATP- and 4-aminopyridine (4AP)-stimulated ethidium bromide fluorescence. The presented results demonstrate, for the first time in healthy, resting human peripheral blood mononuclear cells that 1α,25(OH)$_2$D3 is capable of exerting a rapid, nongenomic effect on $[Ca^{2+}]_i$, while inhibiting of the P2X$_7$ channel permeability.

Keywords: Intracellular calcium; Human lymphocytes; 1α,25(OH)$_2$D3, P2X$_7$ receptor; Fluorescence

1. Introduction

The role of 1α,25(OH)$_2$D3 in the regulation of calcium and phosphorus metabolism is well-known: 1α,25(OH)$_2$D3 receptors are found not only in classical target tissues (intestine, bone, kidney, parathyroid gland), but also in other "nonclassical" tissues, including cells of the immune system such as monocytes as well as activated and resting lymphocytes (Veldman et al., 2000). The steroid hormone produces a number of physiological responses in a variety of cells via both slow, genomic and rapid, nongenomic mechanisms. The genomic action involves high affinity binding to an intracellular vitamin D receptor, followed by an association of the vitamin D receptor complex with vitamin D responsive elements to elicit control of gene expression (Norman and Collins, 1996; Wehling, 1997; Brown et al., 1999). Furthermore, 1α,25(OH)$_2$D3 stimulates a wide array of rapid, nongenomic responses, including changes in phosphoinosite metabolism, increase in $[Ca^{2+}]_i$, activation of protein kinase C (PKC), influx of store-operated calcium influx and opening of voltage-gated calcium and chloride channels (Wehling, 1997). Stimulation of rapid responses by 1α,25(OH)$_2$D3 has been postulated to result from interaction of the ligand with a putative cell membrane receptor for 1α,25(OH)$_2$D3 (Baran et al., 2000; Barsony et al., 1997; Kim et al.,

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1996; Nemere et al., 2000). Concerning the rapid effects of 1α,25(OH)$_2$D3 on [Ca^{2+}]$_i$, both the release from internal stores and the influx from external compartments have been identified, with relative contributions depending on the cell type studied.

Ca^{2+} mobilization is crucial for the activation of lymphocytes. As a part of the immune defence response, antigen binding to surface receptors leads to the generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Elevated levels of IP_3 in the cytosol evoke Ca^{2+} release and depletion from its internal stores (Zweifach and Lewis, 1993; Berridge, 1995), triggering prolonged Ca^{2+} influx through calcium-release activated calcium (CRAC) channels in the plasma membrane (Berridge, 1995; Putny et al., 2001). A sustained increase in cytosolic calcium concentration of about 200 nM up to 1 μM for 48 h is necessary to activate Ca^{2+}-dependent transcription factors: nuclear factor of activated T-cells, Oct/Oap and nuclear factor κB (Lewis, 2001).

Previous studies have shown that voltage-dependent Ca^{2+} channels play an important role in Ca^{2+} mobilization during T-lymphocyte activation (Densmore et al., 1996; Savignac et al., 2001). Kotturi et al. (2003) presented both molecular and pharmacological evidence for the presence of L-type Ca^{2+} channels in the plasma membrane of lymphocytes and hypothesized that L-type Ca^{2+} channels are a significant component of the calcium influx pathways mediating T-lymphocyte activation and proliferation. In skeletal muscle cells (Morelli et al., 2000; Vazquez et al., 1998) and nonexcitable cells, such as osteoblasts (Farach-Carson and Ridall, 1998; Nakagawa et al., 1999) and enterocytes (Lieberherr et al., 1989), 1α,25(OH)$_2$D3 was reported to promote nongenomic Ca^{2+} release from intracellular stores and cation influx through both L-type and store-operated Ca^{2+} channels.

Purinergic $P2X_7$ receptors, known for their affinity to extracellular nucleosides, also intervene in lymphocyte activation. This presence has been demonstrated in cells of immune and inflammatory systems, such as macrophages, lymphocytes, and monocytes (Di Virgilio et al., 2001). These receptors of human lymphocytes produce multiple effects, including ionic fluxes, stimulation of phospholipase D and activation of a membrane protease. Other known functions of $P2X_7$ in the immune response are L-selectin shedding in leukocytes and lymphocytes, ATP-mediated proliferation and apoptosis, killing of intracellular bacteria and activation of intracellular inflammatory-related signal transduction pathways (Jamieson et al., 1996; Fairbairn et al., 2001; Budagian et al., 2003).

In this contribution, we examined the rapid effects of 1α,25(OH)$_2$D3 on calcium mobilization and entry from extracellular sources, including L-type calcium channels and $P2X_7$ receptors, into resting human peripheral blood mononuclear cells isolated from healthy volunteers. We also investigated the possible involvement of purinergic receptors in this action.

2. Materials and methods

2.1. Solutions and drugs

Physiological salt solution-containing in mM NaCl, 140, KCl, 5.4, Na_2HPO_4, 1, $MgCl_2$, 0.5, glucose, 5, and HEPES, 5, titrated to pH 7.4 with NaOH-was supplemented with ethylene glycol-bis(B-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) or Ca^{2+} at different stages of the experimental procedure, as described below.

1α,25(OH)$_2$D3 was purchased from Sigma-Aldrich (Dorset, UK) and thapsigargin (Tg) was procured from Calbiochem (San Diego, CA). 2-Aminoethyldiphenyl borate (2APB), nifedipine, 3′-*O*-(4-benzoyl)benzoyl-adenosine 5′-triphosphate (BzATP), 4-aminopyridine (4AP) and 1-[*N,O*-bis(5-isoquinoline sulfonyl)*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) were obtained from Sigma (St Louis, MO). Fluo-3 acetoxymethylester (Fluo-3 AM) was from Molecular Probes (Eugene, OR), and fetal bovine serum (FBS) and RPMI-1640 medium from GIBCO (Grand Island, NY). Stock solutions were prepared in water (4AP, BzATP), dimethylsulfoxide (Tg, KN-62, Fluo-3 AM) or in ethanol (1α,25(OH)$_2$D3, nifedipine, 2APB). Ethidium bromide (Sigma, St Louis, MO) was prepared in water immediately before use. All other chemicals were purchased from Sigma.

2.2. Cell isolation

Human peripheral blood mononuclear cells were isolated from heparinized blood samples (9 ml) obtained from healthy volunteers according to the procedure acknowledged by the Ethics Committee of the Slovak Medical University. Peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation from blood withdrawn into heparinized syringes, as described previously (Lajdova et al., 2004). Briefly, the samples were diluted 1:1 with RPMI-1640 medium, layered onto an equivalent volume of Histopaque-1077, and centrifuged at 700 g for 20 min at 22 °C. The resulting peripheral blood mononuclear cells layer was washed in 40 ml RPMI, and resuspended in 10 ml RPMI supplemented with 10% FBS. Before loading with a fluorescent probe, the cells were centrifuged at 300 g (10 min at 22 °C), the supernatant was removed, and the pellet was resuspended in 2-ml aliquots of physiological salt solution (see composition above) with 1 mM Ca^{2+}. In this way, we obtained a final concentration of 2×10^6 cells/ml solution. Our preparation contained lymphocytes (90–94%), monocytes (3–6%), and natural killer cells (the rest), as determined by flow cytometry (Coulter EPICS XL, USA). Cell viability was quantified with a 0.8% solution of trypan blue and estimated to be 96–98%.

2.3. Measurement of [Ca^{2+}]$_i$

[Ca^{2+}]$_i$ was measured by using the Ca^{2+}-sensitive fluorescent dye, Fluo-3. Peripheral blood mononuclear cells (2×10^6 cells/ml) were loaded with Fluo-3 AM at a final concentration of 2 μM for 40 min at 22 °C in a physiological salt solution (see Section 2.1 for composition). After incubation, the cells were centrifuged at 300 g, washed 3 times with the physiological salt solution, and kept at room temperature for 10 min before use. Fluo-3 fluorescence was measured at 37 °C in a Fluorolog 3-11 spectrofluorimeter (HORIBA Jobin Yvon Inc., Edison, NJ) with excitation at 488 nm (bandpass 3 nm) and emission at 526 nm (bandpass 5 nm).

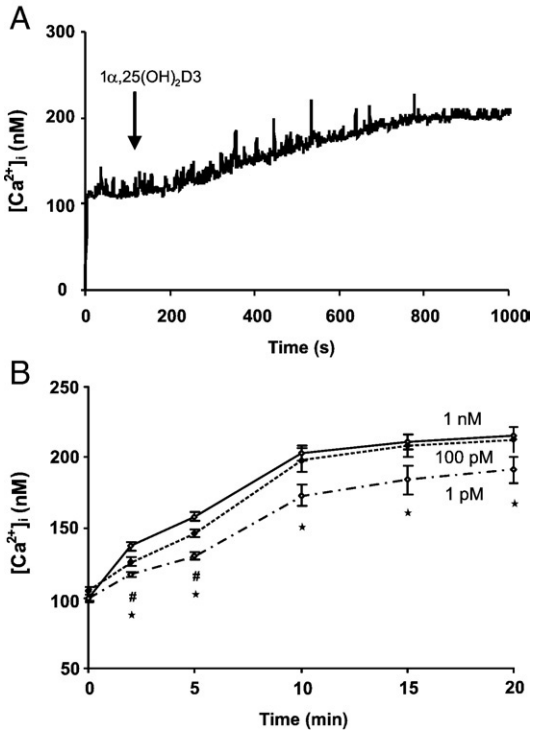

Fig. 1. The steroid hormone $1\alpha,25(OH)_2D3$ induces a two-step response of $[Ca^{2+}]_i$ in resting human peripheral blood mononuclear cells. (A) Effect of $1\alpha,25(OH)_2D3$ (1 nM) in human peripheral blood mononuclear cells loaded with Fluo-3 AM in Ca^{2+}-containing (1 mM) medium. The profile is representative of 8 experiments. (B) Time-dependent effect of $1\alpha,25(OH)_2D3$ (1 nM, 100 pM and 1 pM) on $[Ca^{2+}]_i$ in resting human peripheral blood mononuclear cells. Cells were preincubated with $1\alpha,25(OH)_2D3$ for 0, 2, 5, 10 and 20 min ($n=8$, * $P<0.01$ 1 pM vs. 1 nM, $^{\#}$ $P<0.01$ 100 pM vs. 1 nM).

Each experiment was followed by $[Ca^{2+}]_i$ calibration to estimate the actual free cytoplasmic calcium concentration from the measured fluorescence signal (F) in each cell population. $[Ca^{2+}]_i$ was quantified in nM, as described previously (Lajdova et al., 2004), according to the equation:

$$\left[Ca^{2+}\right]_i = K_d * [(F - F_{min})/(F_{max} - F)]$$

where $K_d=400$ nM at 37 °C (Tsien, 1988). Maximal fluorescence intensity (F_{max}) was assessed by the addition of Triton X-100 (0.1%) with Ca^{2+} (5 mM), and minimum fluorescence level (F_{min}) was determined after the addition of 25 mM EGTA (pH=9). Digitonin (20 μM) was used to answer for minimal compartmentalization (Kao, 1994).

2.4. Estimation of ethidium bromide entry

BzATP, and 4-AP-activated pore functions were measured by using the fluorescent indicator ethidium bromide. Transmission and the fluorescence imaging was done by laser scanning confocal microscope LSM 510 META on an Axiovert 200 inverted microscope (Carl Zeiss, Jena, Germany) using a 40×/1.2 W C-Apochromat water-immersion objective and confocal pinhole opening of 1 Airy unit. A 514 nm Ar:ion laser line served as an excitation source, with a 545-nm dichroic mirror and 560-nm long-pass emission filter for fluorescence detection. Areas measuring 230×230 μm were scanned with 1024×1024-pixel resolution.

2.5. Data analysis

The data are reported as means±S.E.M. The statistical significance of differences was tested by independent 2-population Student's *t* test.

3. Results

3.1. Effect of $1\alpha,25(OH)_2D3$ on $[Ca^{2+}]_i$ levels in resting human peripheral blood mononuclear cells

We examined the effect of $1\alpha,25(OH)_2D3$ on cytoplasmic calcium concentration in healthy human peripheral blood mononuclear cells. $[Ca^{2+}]_i$ was recorded in a population of about 2 millions cells, freshly isolated from healthy volunteers and loaded with Fluo-3 AM. Adopting the calibration procedure described in Materials and methods, $[Ca^{2+}]_i$ was estimated to reach 100.8±1.2 nM under control conditions in the studied ($n=70$) cell populations. Our first goal was to investigate the rapid effects of $1\alpha,25(OH)_2D3$ on calcium mobilization and entry into blood cells. The application of $1\alpha,25(OH)_2D3$ to resting cells induced a sustained increase in $[Ca^{2+}]_i$ (Fig. 1A) under our experimental conditions. We have tested concentrations of the $1\alpha,25(OH)_2D3$ that were in the range of serum

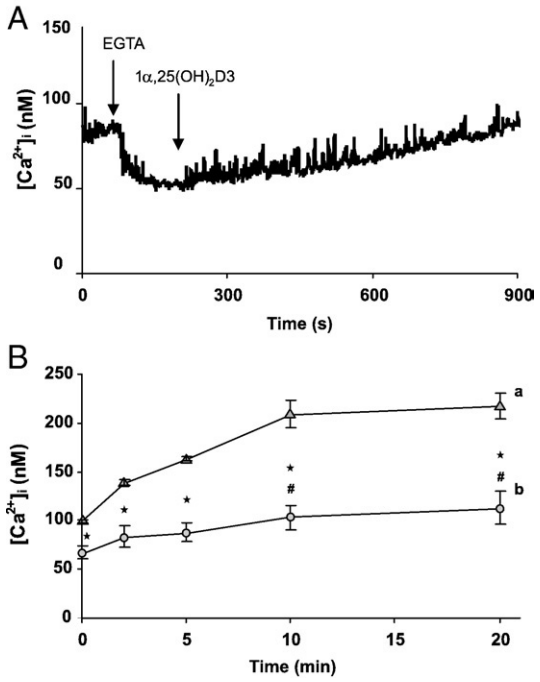

Fig. 2. $1\alpha,25(OH)_2D3$ is responsible for enhanced of Ca^{2+} entry from the external spaces. (A) Typical effect of $1\alpha,25(OH)_2D3$ (1 nM) in the presence of EGTA, a chelator of extracellular calcium, applied in the external medium (pH=7). (B) Time-dependent effect of $1\alpha,25(OH)_2D3$ on $[Ca^{2+}]_i$ in resting human peripheral blood mononuclear cells in the presence (a) and absence (b) of extracellular calcium. Data are means±S.E.M. ($n=8$, $^{\#}$ $P<0.01$ vs. time=0 min, * $P<0.01$ vs. presence of calcium).

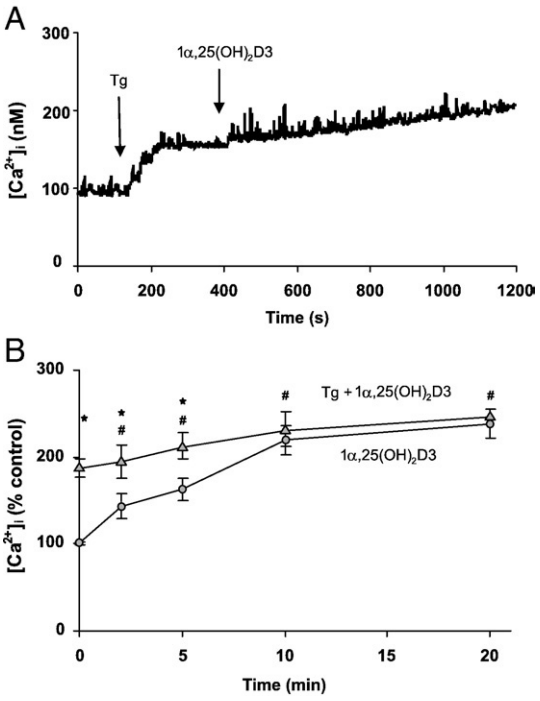

Fig. 3. 1α,25(OH)$_2$D3 induces calcium entry, but not a two-step response in cells where internal stores are emptied by a specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (Tg). (A) Typical experiment where cells were stimulated with 1 μM Tg prior to 1α,25(OH)$_2$D3 application. The profile is representative of 8 experiments. (B) Mean $[Ca^{2+}]_i$ in peripheral blood mononuclear cells treated with 1α,25(OH)$_2$D3 (1 nM) in control conditions and in conditions where intracellular reserves were emptied by Tg. Preincubation time with 1α,25(OH)$_2$D3 varied between 2 and 20 min. Data are means±S.E.M. (n=8, # P<0.01 vs. time=0 min, * P<0.01 vs. absence of Tg).

concentrations in human subjects (10–100 pM) (Panichi et al., 1998; Stefikova et al., 2004). For all tested concentrations of the hormone (1 pM, 100 pM and 1 nM), the calcium rise reached a plateau phase after about 10 min. However, as illustrated in Fig. 1B, while the plateau $[Ca^{2+}]_i$ was significantly increased by 100 pM vs. 1 pM of the hormone, further concentration increment to 1 nM induced only faster response, without additional rise in the plateau calcium levels. We have therefore chosen to investigate the effects of 1α,25(OH)$_2$D3 at the concentration of 1 nM. We observed that after 10-min treatment with 1 nM of 1α,25(OH)$_2$D3, $[Ca^{2+}]_i$ doubled from 99.7±1.4 to 210±14.4 nM (n=8). This effect, corresponding to a 111% increase in cytoplasmic calcium concentration, was significant (P<0.01), and prolonged exposure of the cells to 1α,25(OH)$_2$D3 for 20 and/or 40 min did not induce any further rise in $[Ca^{2+}]_i$ (Fig. 1B).

Interestingly, the hormone also produced an elevation of $[Ca^{2+}]_i$ in human cells in the absence of extracellular calcium chelated with EGTA (pH 7) in the external medium (Fig. 2A). For these experiments, the cells were preincubated with 1 nM of 1α,25(OH)$_2$D3 for 2 to 20 min. In the presence of EGTA, the 1α,25(OH)$_2$D3 effect was time-dependent, but while the increment of $[Ca^{2+}]_i$ was already significant after 5 min in the presence of extracellular calcium, it rose significantly only after 10 min in its absence (Fig. 2B). This observation suggests that although calcium entry from the external compartments contributes to the rapid response of 1α,25(OH)$_2$D3, it is not solely responsible for its action.

Rapid effects of 1α,25(OH)$_2$D3 have been noted in several cell types, but not in human peripheral blood mononuclear cells. We therefore investigated the effect of 1α,25(OH)$_2$D3 on the sustained Ca^{2+} response in these cells under conditions where endoplasmic reticulum Ca^{2+}-ATPase was suppressed by its specific inhibitor, Tg (1 μM). 1α,25(OH)$_2$D3 was applied during the sustained phase of the Tg effect and evoked a further increase in $[Ca^{2+}]_i$ (Fig. 3A). Similar observations on each of the described phenomenon were recorded in at least 8 different populations of human blood cells. Comparison of the time-dependent effects of 1α,25(OH)$_2$D3 in resting cells with functional reserves and those where intracellular Tg-sensitive stores were emptied (Fig. 3B) suggests that the1α,25(OH)$_2$D3-

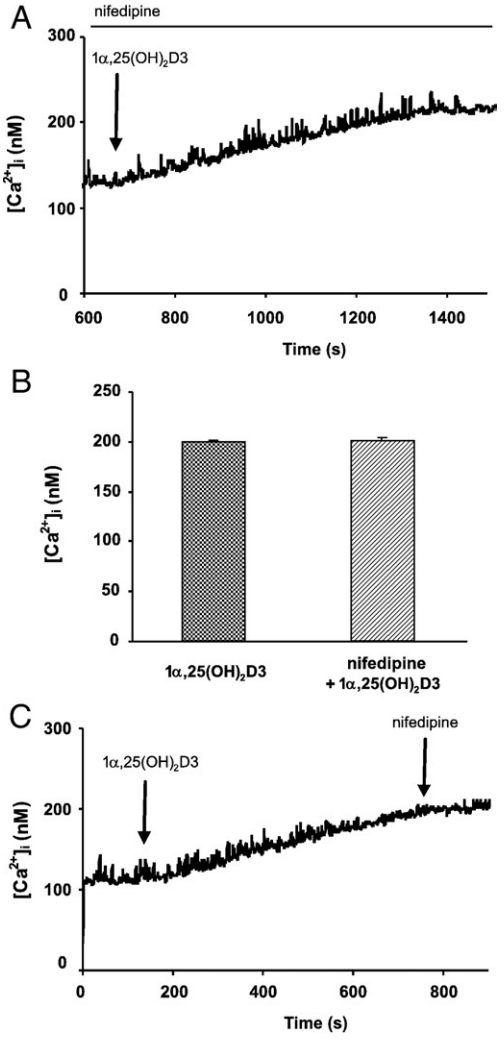

Fig. 4. L-type Ca^{2+} channels are not involved in 1α,25(OH)$_2$D3-induced calcium entry. (A) Effect of 1α,25(OH)$_2$D3 (1 nM) in human peripheral blood mononuclear cells preincubated for 10 min with 100 μM nifedipine, the L-type Ca^{2+} channel dihydropyridine antagonist. (B) Comparison of the effects of 1α,25(OH)$_2$D3 in the absence and presence of 100 μM nifedipine. All values are means±S.E.M. (n=8, ns). (C) Cells were exposed to 1α,25(OH)$_2$D3, followed by the addition of nifedipine (n=8).

stimulated $[Ca^{2+}]_i$ increase results from both calcium release from its internal stores and calcium entry into the cells.

3.2. Calcium entry from extracellular spaces

3.2.1. L-type Ca^{2+} channels

L-type Ca^{2+} channels contribute to Ca^{2+} influx during T-lymphocyte activation and proliferation. To address the question whether L-type Ca^{2+} channels are involved in 1α,25$(OH)_2$D3-induced calcium entry, Fluo-3 AM-loaded human peripheral blood mononuclear cells were treated with nifedipine (100 μM), the dihydropyridine antagonist of L-type Ca^{2+} channels, for 10 min prior to the addition of 1α,25$(OH)_2$D3 (1 nM) (Fig. 4A). Surprisingly, 1α,25$(OH)_2$D3 (1 nM) had a similar effect on $[Ca^{2+}]_i$ in cells pretreated with nifedipine, as it did in the absence of the antagonist (Fig. 4B). Besides, nifedipine had no effect on $[Ca^{2+}]_i$ stimulated by 1α,25$(OH)_2$D3 (see representative trace in Fig. 4C). These results indicate that L-type Ca^{2+} channels are not involved in 1α,25$(OH)_2$D3-induced calcium entry.

3.2.2. Capacitative calcium entry

The central idea of the capacitative regulation of $[Ca^{2+}]_i$ is that the refilling of cytosolic Ca^{2+} reserves terminates Ca^{2+} entry. These channels can, therefore, also contribute to the observed effect of 1α,25$(OH)_2$D3 in peripheral blood mononuclear cells.

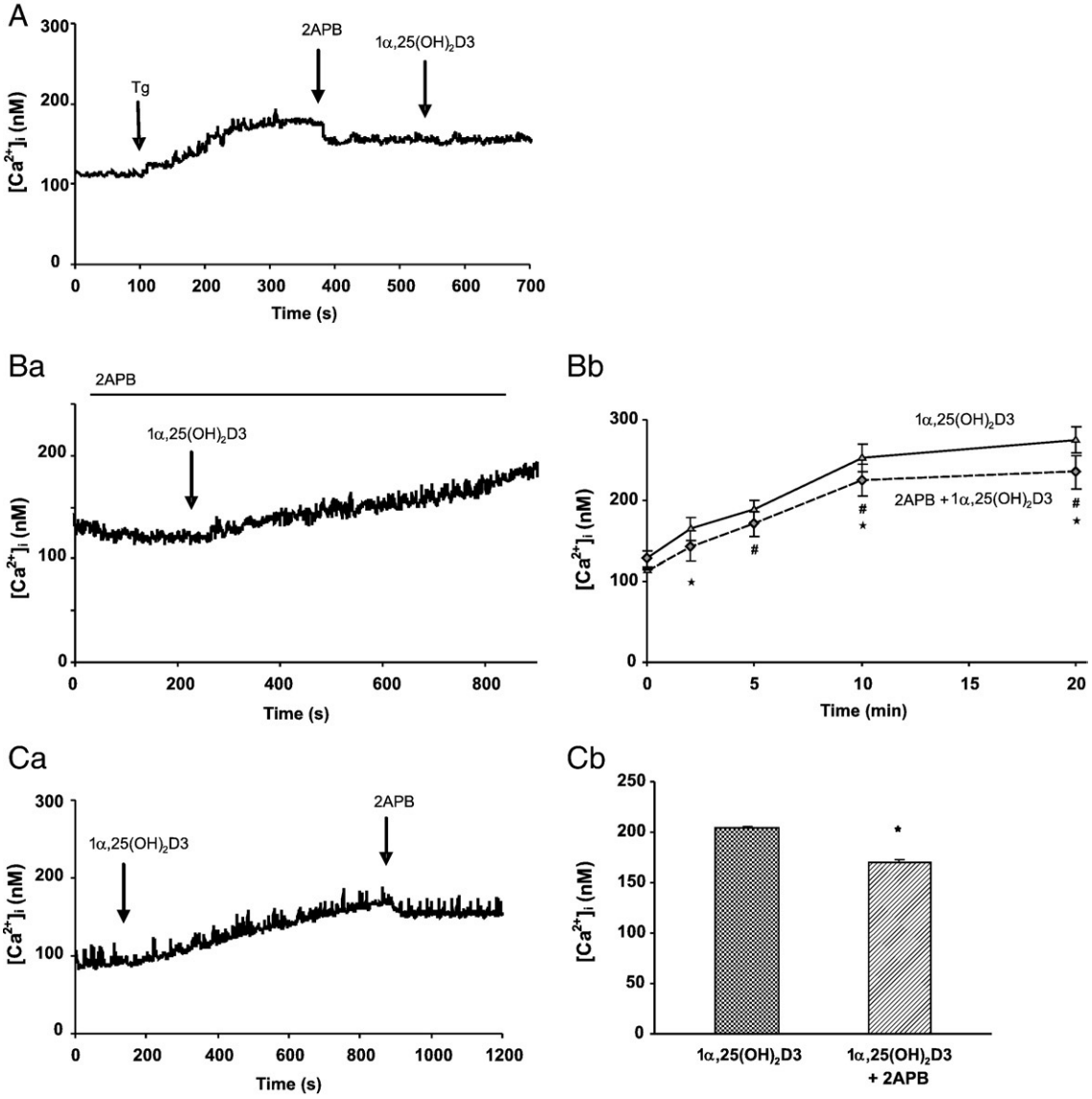

Fig. 5. 1α,25$(OH)_2$D3 induces capacitative Ca^{2+} influx in resting human peripheral blood mononuclear cells. (A) Effect of 1α,25$(OH)_2$D3 after activation of CRAC channels by intracellular Ca^{2+} store depletion by Tg (1 μM) and their subsequent inhibition by 2APB. The profile is representative of 8 experiments. (Ba) Cells were exposed to 1α,25$(OH)_2$D3 (1 nM) after the addition of 50 μM 2APB, the inhibitor of capacitative calcium entry. The profile represents 8 experiments. (Bb) Comparison of the time-dependent effects of 1α,25$(OH)_2$D3 in resting human peripheral blood mononuclear cells and cells pretreated with 2APB for 10 min prior to application of 1α,25$(OH)_2$D3. Data are means±S.E.M. ($n=8$, $^{\#}$ $P<0.01$ vs. time=0 min, * $P<0.01$ vs. absence of 2APB). (Ca) Effect of 2APB on 1α,25$(OH)_2$D3 treated cells. The profile is representative of 8 experiments. (Cb) Effect of 2APB on the 1α,25$(OH)_2$D3 action (1 nM for 10 min). All values are means±S.E.M. ($n=8$, * $P<0.01$).

To examine the possible participation of capacitative calcium entry in the action of 1α,25(OH)$_2$D3, we tested 2APB. Originally described as a membrane-permeable inhibitor of IP$_3$ receptors (IP$_3$Rs) (Maruyama et al., 1997), 2APB was also demonstrated to inhibit capacitative calcium entry channels at their extracellular sites by an IP$_3$Rs-independent mechanism (Bootman et al., 2002). The inhibitory action of 2APB (50 μM) was verified in cells where capacitative calcium entry was stimulated by Tg (1 μM) (see example in Fig. 5A). Subsequent application of 1α,25(OH)$_2$D3 had no impact on $[Ca^{2+}]_i$. In parallel, in cells pretreated with 2APB (50 μM, 10 min), we noted that 1α,25(OH)$_2$D3 application still caused a rise in $[Ca^{2+}]_i$ (Fig. 5Ba), but this effect was significantly smaller when compared to that of 1α,25(OH)$_2$D3 alone (Fig. 5Bb). Similar observations were recorded in 8 different populations of human blood cells. In addition, application of 2APB (50 μM) on resting cells treated for 10 min with 1α,25(OH)$_2$D3 caused a significant decrease in $[Ca^{2+}]_i$ (Fig. 5Ca and Cb). The reported findings strongly suggest that 1α,25(OH)$_2$D3 provokes the stimulation of capacitative calcium entry.

3.3. Purinergic P2X$_7$ receptors

Purinergic P2X$_7$ receptors intervene in lymphocyte activation via multiple processes, including calcium influx and the permeation of large cations. We, therefore, investigated the possible involvement of these receptor channels in the 1α,25(OH)$_2$D3-induced effect in resting human peripheral blood mononuclear cells. P2X$_7$ receptors are activated by BzATP, partially stimulated

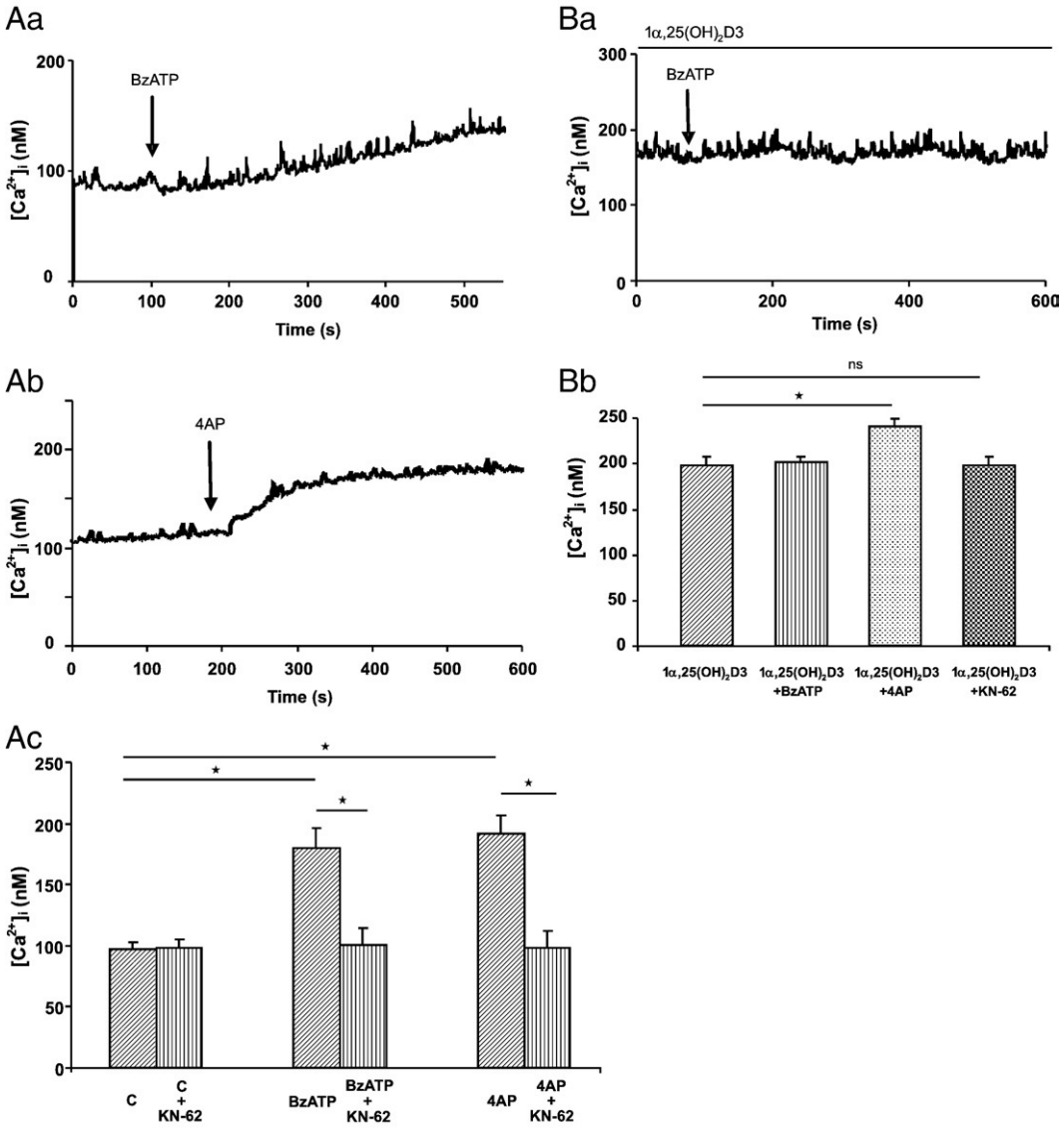

Fig. 6. The steroid hormone 1α,25(OH)$_2$D3 inhibits calcium influx through P2X$_7$ channels. (Aa) Cells exposed to 50 μM BzATP, a specific agonist of P2X$_7$ receptors and (Ab) 5 mM 4AP, a K^+ channel blocker and activator of calcium influx through P2X$_7$ channels. The profiles are representative of 8 experiments. (Ac) Cytoplasmic calcium concentrations in cells exposed to 50 μM BzATP, 5 mM 4AP or 1 μM KN-62. All values are means ± S.E.M. (n = 10, * P < 0.01). (Ba) Typical effect of BzATP (50 μM) in the presence of 1 nM 1α,25(OH)$_2$D3. The profile is representative of 8 experiments. (Bb) Application of 50 μM of the specific purinergic receptor agonist BzATP failed to increase $[Ca^{2+}]_i$ in the presence of 1α,25(OH)$_2$D3. Also, while 5 mM 4AP induced some rise in $[Ca^{2+}]_i$ in the presence of the steroid hormone, 1 μM KN-62 failed to affect the $[Ca^{2+}]_i$ rise induced by 1α,25(OH)$_2$D3. The data are means ± S.E.M. (n = 8, * P < 0.01).

by high extracellular ATP concentrations (Gargett et al., 1997), and specifically inhibited by KN-62 (Gargett and Wiley, 1997). To test the implication of $P2X_7$, BzATP (50 μM), a specific purinergic receptor agonist, was added to the external media. While BzATP-induced a significant (84%, $P<0.01$) rise in $[Ca^{2+}]_i$ (to 187± 8.7 nM) in the absence of the hormone (Fig. 6Aa and Ac), it failed to increase $[Ca^{2+}]_i$ in cells pretreated for 10 min with 1α,25 $(OH)_2D3$ (1 nM) (Fig. 6Ba and Bb).

Comparably, the specific inhibitor of purinergic $P2X_7$ receptors KN-62 also failed to affect 1α,25$(OH)_2D3$-activated calcium entry (Fig. 6Ac and Bb). In our previous work we have shown that 4AP, a nonspecific inhibitor of potassium channels, induced a rise of $[Ca^{2+}]_i$ which was mediated by $P2X_7$ receptors (Lajdova et al., 2004). Therefore, the effect of 1α,25$(OH)_2D3$ on 4AP-induced calcium influx was also investigated. 4AP (5 mM) significantly increased $[Ca^{2+}]_i$ (Fig. 6Ab and Ac), but the effect was reduced in the presence of 1α,25$(OH)_2D3$ (data not illustrated), although the effect of 4AP on $[Ca^{2+}]_i$ still remained significant (Fig. 6Bb). Our findings point to the fact that 1α,25 $(OH)_2D3$ prevents the opening of purinergic $P2X_7$ receptors and thus calcium entry through this nonspecific cationic channel.

To provide further proof of purinergic channel involvement in the regulation of 1α,25$(OH)_2D3$ action, we also investigated its pore size. The ability to form membrane pores that are permeable to large cations, including fluorescent dyes, is a specific property of $P2X_7$ receptors. In lymphocytes, these pores are permeable to molecules up to 320 Da (Wiley et al., 1993), such as ethidium bromide (314 Da). Ethidium bromide permeability through BzATP- or 4AP-activated channels in the absence and presence of 1α,25$(OH)_2D3$ (1 nM) was examined by fluorescence measurements. While nearly no ethidium bromide fluorescence was seen in control cells or cells pretreated for 10 min with 1α,25$(OH)_2D3$ (1 nM) (Fig. 7A and B), as expected, the application of 50 μM BzATP and 5 mM 4AP stimulated the entry of ethidium bromide ions into human peripheral blood mononuclear cells (Fig. 7C and E). However, 1α,25$(OH)_2D3$ decreased the BzATP- and 4AP-induced fluorescence intensity of ethidium bromide (Fig. 7D and F). After quantification (Fig. 7G), comparison of ethidium bromide fluorescence intensities of the tested cells pointed to an inhibitory effect of 1α,25$(OH)_2D3$ on calcium influx through $P2X_7$ receptors.

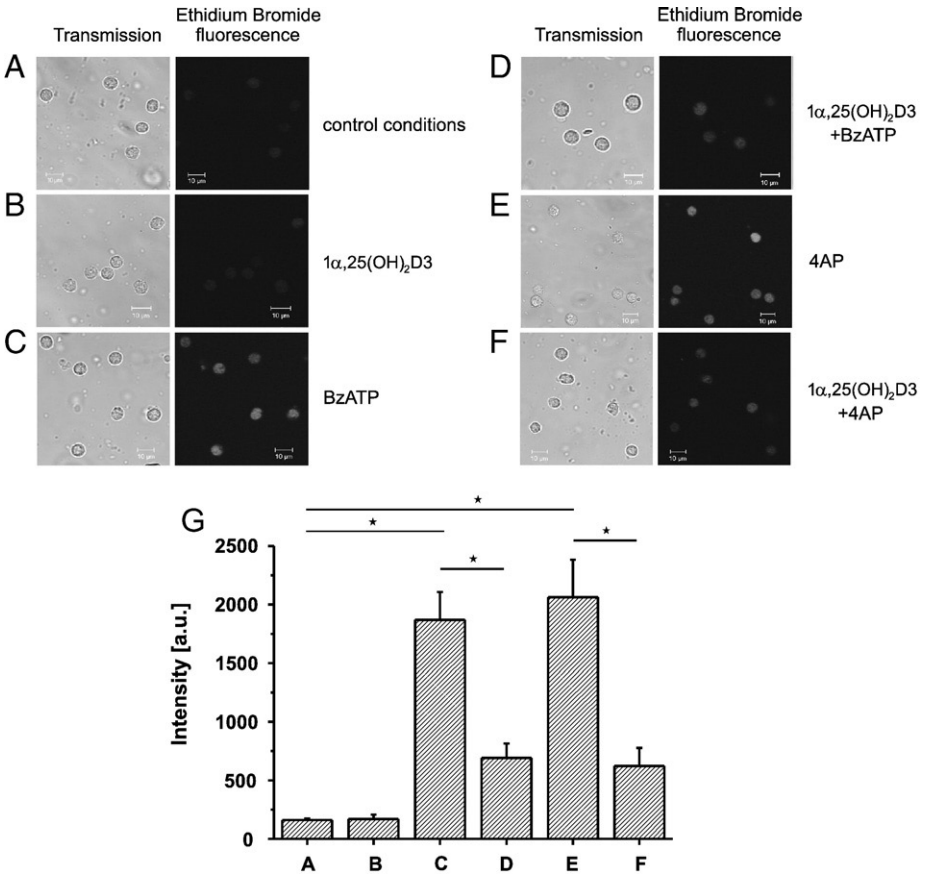

Fig. 7. 1α,25$(OH)_2D3$ reduces ethidium bromide fluorescence in cells after the addition of BzATP and 4AP. Ethidium bromide was added to cell suspensions 30 s before BzATP or 4AP. The fluorescence images were taken after 8-min exposure to BzATP or 4AP. The transmission images (left) and ethidium bromide (30 μM) fluorescence (right) are shown for human peripheral blood mononuclear cells in (A) control condition, (B) after pretreatment with 1 nM 1α,25$(OH)_2D3$ for 10 min (C) stimulation with 50 μM BzATP, (D) treatment with 1α,25$(OH)_2D3$ for 10 min prior to BzATP (50 μM) application, (E) stimulation with 5 mM 4AP for 8 min, (F) pretreatment with 1 nM 1α,25$(OH)_2D3$ for 10 min, followed by 4AP (5 mM) for 8 min, and (G) comparison of the ethidium bromide fluorescence intensities of tested cell populations ($n=6$,* $P<0.01$).

4. Discussion

In this contribution, we provide evidence that 1α,25 $(OH)_2$D3 is capable of inducing rapid nongenomic responses in resting human peripheral blood mononuclear cells. We demonstrate that the steroid hormone provokes a two-step response: an initial 1α,25$(OH)_2$D3-stimulated Ca^{2+} mobilization from intracellular Tg-sensitive stores, followed by a sustained Ca^{2+} influx from the extracellular spaces through CRAC channels but not L-type calcium channels. Interestingly, we also observed nongenomic inhibitory actions of 1α,25 $(OH)_2$D3 on calcium influx through $P2X_7$ receptors, which are known to be involved in inflammation and apoptosis (Humphreys et al., 2000; Fairbairn et al., 2001). To our knowledge, this is one of the first reports of such effects of 1α,25$(OH)_2$D3 in resting human peripheral blood mononuclear cells.

In recent years, substantial efforts have been made to understand the possible role of 1α,25$(OH)_2$D3 and its analogues in the immune system and organ transplantion (Veldman et al., 2000; DeLuca and Cantorna, 2001). The immunomodulatory effects of 1α,25$(OH)_2$D3 have been studied in lectin- and antigen-activated lymphocytes as well as in autoimmune disease models (such as experimental autoimmune encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, type I diabetes and inflammatory bowel disease). These contributions showed that 1α,25$(OH)_2$D3 affects cells genomically through a nuclear receptor and/or nongenomically via membrane receptors. A crucial finding of our investigation is that 1α,25$(OH)_2$D3 induces a time-dependent increase in $[Ca^{2+}]_i$ which remains stable after 10 min. We demonstrate that pretreatment of cells with 1α,25$(OH)_2$D3 (10 min) provoked Ca^{2+} mobilization from intracellular Tg-sensitive stores. A presence of sustained Ca^{2+} increase in cells where internal stores were emptied by Tg indicated that 1α,25$(OH)_2$D3 affected not only Tg-sensitive stores, but also influx from the extracellular spaces and/or other intracellular calcium compartments.

Our results showed that the rapid calcium increase induced by 1α,25$(OH)_2$D3 was followed by sustained Ca^{2+} flux, which was abolished in the absence of extracellular calcium. Partial inhibition of the 1α,25$(OH)_2$D3 effect in the presence of EGTA strongly indicates that 1α,25$(OH)_2$D3 induces calcium influx in addition to IP_3-stimulated calcium release from intracellular stores. We, therefore, studied the possible contributors to this calcium entry. Previous studies have implicated L-type Ca^{2+} channels in Ca^{2+} influx during T-lymphocyte activation and proliferation (Brix et al., 1984). Nonexcitable cells express Ca^{2+} channels that share common structural features with voltage-dependent Ca^{2+} channels of electrically-excitable cells but are not solely gated by changes in membrane potential (Densmore et al., 1996). Several pharmacological investigations have provided further data to support the expression of voltage-dependent Ca^{2+} channels in T-lymphocytes (Kotturi et al., 2003). Furthermore, studies on the rapid 1α,25$(OH)_2$D3-induced elevation of $[Ca^{2+}]_i$ levels in osteoblasts (Farach-Carson and Ridall, 1998; Nakagawa et al., 1999) as well as in skeletal and cardiac muscle cells (Falkenstein et al., 2000) have demonstrated that the effect of the hormone comprised both Ca^{2+} release from intracellular stores and extracellular Ca^{2+} influx via an L-type Ca^{2+} channels. However, despite the importance of these channels in lymphocytes, our experiments with nifedipine revealed that L-type Ca^{2+} channels do not contribute to the Ca^{2+} rise stimulated by 1α,25$(OH)_2$D3. Besides, observations that 2APB fully inhibited Tg-stimulated capacitative entry and that 1α,25$(OH)_2$D3 completely lacked the effect on $[Ca^{2+}]_i$ in this setting both support the fact that in peripheral blood mononuclear cells, calcium entry through L-type Ca^{2+} channels is not observable and thus not implicated in the effect of the hormone. This is true even when CRAC channels are closed, in agreement with findings already reported by others (Braun et al., 2003).

Calcium entry into nonexcitable cells is known to be mediated via store-operated Ca^{2+} channels and triggered by Ca^{2+} depletion from intracellular stores. Ca^{2+} influx through CRAC channels is essential for lymphocyte activation and proliferation. It activates several transcription factors, which regulate the expression of cytokine genes for the immune response (Lewis, 2001; Putny et al., 2001). We, therefore, applied the inhibitor of CRAC channels to study their involvement in the 1α,25$(OH)_2$D3 action in peripheral blood mononuclear cells. 2APB, an inhibitor of capacitative calcium entry (Bootman et al., 2002), caused a significant decrease in $[Ca^{2+}]_i$ in peripheral blood mononuclear cells treated with 1α,25$(OH)_2$D3, pointing to the role of CRAC channels in the 1α,25$(OH)_2$D3 action.

$P2X_7$ receptors exist in two functionally-distinct states, one of which corresponds to a channel that opens rapidly in response to agonists and passes small inorganic cations such as Ca^{2+}. With prolonged agonist application, these receptors form pores capable of passing larger organic ions such as ethidium bromide. Our data disclosed that 1α,25$(OH)_2$D3 inhibited calcium influx through $P2X_7$ channels and reduced pore permeation. The presence of purinergic receptors of the $P2X_7$ type was demonstrated in human lymphocytes (Gargett et al., 1997) as well as in other blood cells (Sluyter et al., 2001; Di Virgilio et al., 2001). $P2X_7$ receptor channels are activated by BzATP and are partially stimulated by high extracellular ATP concentrations (Gargett et al., 1997). In our previous work (Lajdova et al., 2004), we showed that the rise in $[Ca^{2+}]_i$ triggered by 4AP was also mediated by $P2X_7$ receptors. The 4AP, originally known for its capacity to inhibit K_v channels and to provoke membrane depolarization, was also linked to the activation of calcium influx. The others authors (Faria et al., 2005) demonstrated that intracellular Ca^{2+} is necessary for the pore formation associated with $P2X_7$ receptors. Data presented in this contribution suggested that 4AP- and BzATP-induced activation of $P2X_7$ receptors was blunted by the steroid hormone. Our results are in accordance with the study of Cario-Toumaniantz et al. (1998), who provided the first evidence of steroid hormone effects on $P2X_7$ receptors, showing that the steroid hormone 17β-oestradiol directly inhibits cation current through human $P2X_7$ receptors and might use this pathway to modify the functional properties of cells expressing these receptors. Taking into consideration our earlier findings that 4AP stimulates the Ca^{2+} rise through $P2X_7$ channels and the discovery of inhibition of Ca^{2+} influx triggered by 4AP (Barbar et al., 2003), we propose that PKC can be one mechanism underlying the action of 1α,25

$(OH)_2D3$ on $P2X_7$ receptors. Lately, the role of PKC in the regulation of $P2X_7$ receptor-mediated Ca^{2+} signalling has been studied intensively (Bradford and Soltoff, 2002). Rosado and Sage (2000) demonstrated that the mechanism for receptor-evoked Ca^{2+} entry in human nonexcitable cells is independent of Ca^{2+} store depletion, but may be directly activated by PKC. Previous studies (Slater et al., 1995) also showed PKC direct activation by $1\alpha,25(OH)_2D3$. Nevertheless, further work is needed to determine whether PKC is involved in the effect of the hormone on $P2X_7$ channels in peripheral blood mononuclear cells. Until recently, it was thought that $P2X_7$ was the P2X receptor mainly expressed by immune cells, but more recent reports suggest the presence of $P2X_1$ and $P2X_4$ receptors (Di Virgilio et al., 2001). In the present work, we have not examined other P2X subtypes expressed in healthy human peripheral blood mononuclear cells. In recent years, $P2X_7$ receptors became the centre of interest, and their possible role is now elucidated in inflammation, hypertension, osteoporosis and kidney diseases (Di Virgilio and Sollini, 2002; Jorgensen et al., 2002; Hillman et al., 2005). In this context, the action of $1\alpha,25(OH)_2D3$ on these receptors would become an attractive potential target for anti-inflammatory drugs. Nevertheless, it is noteworthy that our observation is just the first step in understanding the possible participation of P2X receptors in the action of $1\alpha,25(OH)_2D3$. A thorough investigation is needed to clarify in further detail, the role of these receptors in the action of the hormone.

In conclusion, in this contribution, we demonstrated that the steroid hormone $1\alpha,25(OH)_2D3$ is capable of inducing rapid, nongenomic increases in $[Ca^{2+}]_i$ in healthy human peripheral blood mononuclear cells. Our findings revealed that $1\alpha,25(OH)_2D3$ promotes a two-step calcium response: a fast, transient rise in $[Ca^{2+}]_i$ due to store depletion, followed by a sustained phase corresponding to store refilling via activation of the CRAC influx pathway, but not L-type calcium channels. Furthermore, $1\alpha,25(OH)_2D3$ prevented the calcium entry induced by BzATP, an agonist of $P2X_7$ receptors, and reduced 4AP-stimulated Ca^{2+} increase. $1\alpha,25(OH)_2D3$ also reduced BzATP- and 4AP-stimulated ethidium bromide fluorescence, confirming the inhibitory effect of $1\alpha,25(OH)_2D3$ on calcium influx through $P2X_7$ channels. Nevertheless, further study is needed to fully explore the nongenomic outcomes of $1\alpha,25(OH)_2D3$ in pathophysiological conditions and their possible therapeutic benefits.

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