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Potassium currents and effects of vitamin D-3 metabolites and cyclic GMP in rat osteoblastic cells

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A K^+ current (I_{K1}), activated by depolarization above −20 mV, showing voltage-dependent inactivation within a few seconds and reduced by 40% by 1 mM TEA, was observed in all cells. In a few cells, we also observed a progressive K^+-current increase during cell dialysis. The developping current (I_{K2}) was not sensitive to 1 mM TEA and did not inactivate. It was detectable over the whole voltage range and slowly increased during 10 s depolarizations. 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ did not affect I_{K1}, but induced a small K^+-current increase in some cells showing no I_{K2}. This effect was not mimicked by cyclic GMP analogs which, on the contrary, induced a K^+-current decrease. 24,25-$(OH)_2D_3$ (even at 10^{-11}M), but not 1,25-$(OH)_2D_3$, strongly reduced I_{K2}. The results further document the diversity of voltage-gated currents of osteoblastic cells, confirm the existence of immediate effects of vitamin D-3 metabolites, which are independent of classical 1,25-$(OH)_2D_3$ receptors.

Introduction

Recently, it has been shown in several cell types that 1,25-$(OH)_2D_3$, in addition to inducing slowly-developing, classical steroid-like receptor-mediated nuclear effects, may induce early effects, occurring within seconds or minutes. These early effects include: (1) an increase of Ca^{2+}-uptake in epithelial intestinal cells [1]; (2) an increase of intracellular Ca^{2+} in various cell types [2–12]; (3) a stimulation of phosphoinositide breakdown in keratinocytes [8,13], rat enterocytes [14] and rat colonic epithelial cells [9]; (4) an increase in intracellular cyclic GMP in human skin fibroblasts [15,16] and (5) modifications of the membrane potential of cartilage and renal cells [17–19], and the modulation of the dihydropyridine-sensitive calcium current of osteosarcoma cells [20].

The rapidity of these effects suggests that they are non-genomic. However, some of them seem to require the presence of functional classical vitamin D receptors [14,15], whereas others can be observed in cells lacking these receptors [12]. The classical vitamin D receptor has a much higher affinity for 1,25-$(OH)_2D_3$ than for another, more abundant, metabolite, 24,25-$(OH)_2D_3$, so that the observation of effects of low concentrations of 24,25-$(OH)_2D_3$ would indicate that classical vitamin D receptors are not involved.

Vitamin D has a dual action on bone: it induces an antirachitic effect leading to enhanced mineralisation, and it causes an increase in bone resorption. Osteoblasts are target cells for 1,25-$(OH)_2D_3$. This hormone enhances the synthesis of various osteoblast-produced factors, among which osteocalcin [21]. It also stimulates osteoblasts to release a soluble factor that increases osteoclastic bone resorption [22]. 1,25-$(OH)_2D_3$ also seems to have a direct stimulatory effect on the in-vitro mineralisation induced by an osteoblastic-like cell line [23].

We have investigated the early effect of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ on the ionic currents of cultured rat osteoblasts. The present paper describes the potassium currents of these cells, measured using the whole-cell configuration of the patch-clamp technique, and reports some effects of vitamin D metabolites on these currents. Since certain studies [15,24] have shown that vitamin D metabolites cause an increase in intracellular cyclic GMP in other cell types, we also investigated the effect of cyclic GMP analogs.

The results indicate that osteoblasts show different types of potassium currents activated by membrane depolarization. Whereas all cells showed a slowly inactivating K^+ current that remained stable with successive depolarizing jumps, some cells also showed an additional K^+ current (I_{K2}), that spontaneously in-

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creased in amplitude during dialysis. We show that in cells where I_{K2} did not develop, the total potassium current could be slightly increased reversibly, by both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, whereas it was reduced by cyclic GMP analogs. We also show that 24,25-$(OH)_2D_3$ (even at 10^{-11} M), but not 1,25-$(OH)_2D_3$, can reduce I_{K2}. The selectivity of this effect of 24,25-$(OH)_2D_3$ supports the controversial proposal that this abundant metabolite may have a specific role in bone.

Materials and Methods

The experiments were performed at room temperature on primary cultures of newborn rat osteoblasts, using the whole-cell configuration of the patch-clamp technique [25].

Cell preparation

Cell preparation was as described in Ref. 26. Osteoblastic cells were isolated from newborn rat calvaria. The central parts of parietal bones were excised and the periosteal tissues carefully stripped away in order to eliminate chondrocytes, suture cells and periosteal progenitor cells. Bones were incubated at 37°C during two sequential 10-min periods in a Ca^{2+}/Mg^{2+}-free Earle solution containing 0.5 mg trypsin/ml (Worthington) and 4 mM EDTA. Isolated cells were harvested, washed and seeded at about 10000 cells/dish (35 mm Falcon) in BGJ medium (Flow Laboratories) supplemented with 10% fetal calf serum (Flow Laboratories), fungizone (2.5 μg/ml), penicillin (100 iu/ml) and streptomycin (50 μg/ml). Experiments were performed on isolated cells from day 4 to day 7 in culture.

Solutions

The culture dish was usually perfused with an external solution containing, in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid)-NaOH (pH 7.4). In a few experiments, we used a high-K^+ external solution, containing 148 mM KCl instead of the usual 140 mM NaCl and 5 mM KCl. A two- (or three-) barrel fast-perfusion system (made of glass and teflon tubing) was used for rapid application of hormones or pharmacological agents. One of the barrels was filled with the control external solution, the others with the test solutions. The recorded cell was continuously perfused with one of these barrels (solutions flowing by gravity), and the fast perfusion system was moved laterally in order to apply the desired solution onto the cell. Stock solutions of 10^{-5} M 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ in absolute ethanol were kept in the dark at −20°C under N_2. Sequential dilutions were performed in the external solution just before use. The control barrel and the test barrel of the fast perfusion system always contained the same ethanol dilution (max. 0.01%). A separate fast perfusion system was used for each substance to be tested, in order to avoid possible contamination by hormone sticking to the perfusion system. The pipette was usually filled with a solution containing, in mM: 141 KCl, 1 $MgCl_2$, 0.1 EGTA (ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid), 3 Na_2ATP and 10 Hepes (pH 7.3). In a few experiments, we used a low-Cl^- internal solution, containing 11 mM KCl and 128 mM K glutamate instead of the usual 141 mM KCl; in this case the junction potential between the electrode and the bath was measured and taken into account.

Recording and analysis

Patch-clamp micro pipettes were made from hard-glass (Kimax 51); the shank of each pipette was covered with Sylgard and the tip was fire-polished. The resistance of the electrodes filled with the KCl internal solution was between 5 and 7 MΩ. The cells were voltage-clamped by an EPC7 List amplifier, controlled by a Tandon 38620 computer, via a Cambridge Electronic Design (CED) 1401 interface, using CED patch- and voltage-clamp software. The current monitor output of the amplifier was filtered at 0.1 kHz before being sampled on-line at 0.2 kHz. Voltage jumps were regularly applied and the current recorded during depolarizing jumps was measured with respect to the zero-current level. We frequently observed the development of an inward current at −100 mV during the first minutes of the whole-cell recording; usually, this inward current gradually disappeared. Spontaneous changes of this current were not correlated with any current change at 0 mV and thus did not interfere with the study of K^+ currents at 0 mV. However, in order to study K^+ currents below or above 0 mV, it was necessary either to wait long enough so that the inward current recorded at −100 mV spontaneously disappeared (Figs. 1, 6, 7B and 8), or to add a blocker of this inward current (Fig. 4A) or to use a low-Cl^- internal solution (Fig. 4B) which was found to prevent the spontaneous changes of this current (Chesnoy-Marchais, D. and Fritsch, J., data not shown).

The series resistance was systematically measured several times during each experiment. Particular care was taken to avoid the use of experiments in which the series resistance changed over time (we were always looking at the capacitive current on an oscilloscope before filtering for sampling). In the experiments selected for this study, the series resistance was between 10 and 13 MΩ. Even though these values are high enough to introduce a difference of a few millivolts between the voltage applied to the electrode and that actually applied to the inside of the cell, the error occurring for a repetitive voltage-jump was constant

throughout each experiment and cannot account for the pharmacological or hormonal modulations described, which furthermore, were shown to be reversible. The use of larger electrodes (of lower resistance) was excluded, since an extensive exchange of the intracellular medium by the internal solution present in the electrode induced a large outwardly rectifying chloride current and prevented a reliable study of other currents (personal observations; see Ref. 27 for similar observations in the same cells using a different internal solution; see also Refs. 28 and 29 showing that epithelial cells contain a cytosolic chloride-channel inhibitor). The smallest capacitance of the cells recorded in the present study was 60 pF and the highest values were slightly superior to 100 pF. $I-V$ curves were performed on the cells which showed both the smallest capacitance and the lowest series resistance.

Results

I_{K1}

Fig. 1A illustrates the current traces recorded in voltage-clamp during 10-s depolarizing jumps from -100 mV to one of the following voltage levels: -30, -10, $+10$, $+30$ mV. On such a slow time-scale, in most cells, the main current is a voltage-sensitive outward current, which activates with depolarization during the first few hundred ms and then slowly inactivates. The observation that this current is outwardly directed in the voltage-range illustrated (on either side, and even at the chloride equilibrium potential, which is 0 mV with the solutions used), indicates that it is carried by K^+ ions. We also observed the reversal of this current at 0 mV when using a K^+-rich external solution bringing E_K to 0 mV (see Fig. 4Bb). We called

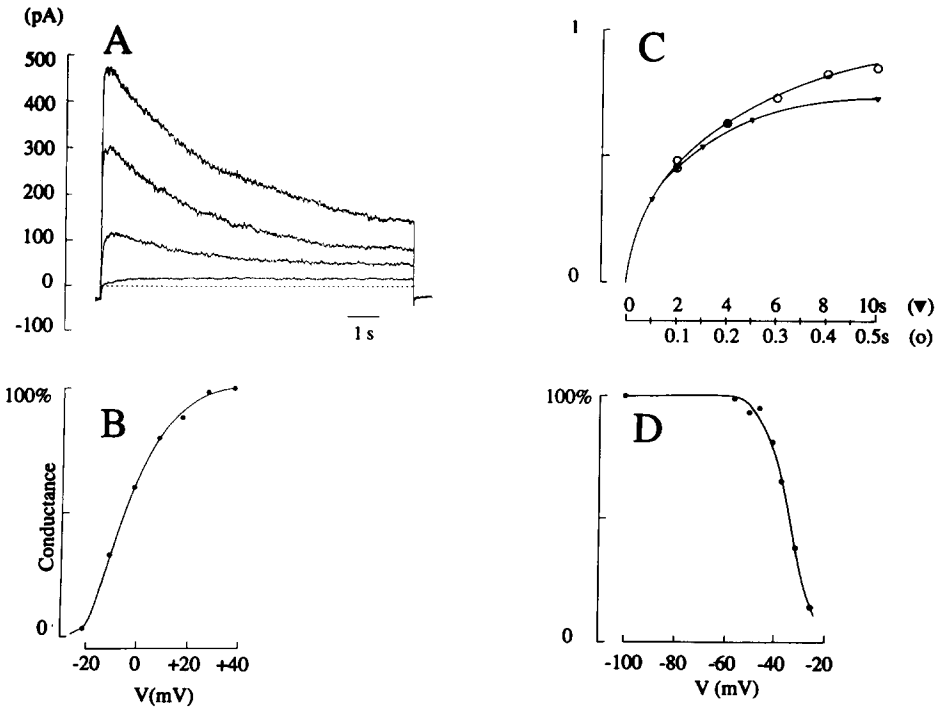

Fig. 1. Depolarizing jumps activate a slowly-inactivating K^+ current, I_{K1}. (A) Current traces recorded during 10 s depolarizing jumps from -100 mV to -30, -10, $+10$ and $+30$ mV. (B) Activation curve of the conductance underlying I_{K1} (same cell as in A). For each membrane depolarization from -100 mV to the potential V, the difference between the peak current and the current measured at end of the depolarization was divided by the driving force for K^+ ions, $V-E_k$, where E_k is the K^+ equilibrium potential (-81 mV in this experiment). This conductance (expressed as a percentage of its maximum value, obtained for a depolarization from -100 to $+40$ mV) is given as a function of V. Half-activation is observed close to -6 mV. In this experiment, the internal solution contained, in mM, 127 KCl, 14 NaCl, 1 $MgCl_2$, 0.1 EGTA, 3 MgATP and 10 Hepes-NaOH (pH 7.3). (C) Inactivation recovery curves for two holding potentials: -50 mV and -100 mV. Note that two time scales, differing by a factor of 20, have been used. The cell was held either at -50 mV (▼) or at -100 mV (○) and pairs of depolarizing jumps to 0 mV, separated by a variable time interval indicated on the horizontal axis, were applied. Between each pair of depolarizations, the cell was maintained at the holding potential long enough for complete inactivation recovery (10 s at -100 mV, 60 s at -50 mV). The ratio between the peak-current values recorded at 0 mV during the second and first depolarization of each pair is plotted on the vertical axis. Half-recovery required 120 ms at -100 mV and 2.5 s at -50 mV. (D) Inactivation curve of I_{K1} (same cell as in A and B). A series of depolarizing jumps to 0 mV from various holding potentials was applied, and for each depolarization to 0 mV, I_{K1} was measured as the difference between the peak outward current and the current value at the end of the depolarization. This difference (expressed as a percentage of its maximum value, obtained for a holding potential of -100 mV) is given as a function of the holding potential. The cell was maintained at each holding potential for 50 s before being depolarized to 0 mV. Between each holding potential test, the cell was maintained at -100 mV for 10 s, in order to allow the current to recover from inactivation.

this inactivating K^+ current I_{K1}, and we estimated it for each depolarization by measuring the difference between the peak outward current and the current remaining at the end of the 10-s depolarization.

Such a current has already been observed in some chick osteoblasts [30]; however, it was only rarely detected [31], whereas in our studies, I_{K1} was observed in almost all the cells.

Fig. 1B gives the activation curve of the K^+-conductance underlying I_{K1}. In three similar experiments, half-activation was observed at -7 ± 0.8 mV (mean $\pm \sigma_n$).

Fig. 1C illustrates the recovery from inactivation of the K^+-current induced by 10-s depolarizations to 0 mV, for two holding potentials: -100 mV and -50 mV. When the holding potential was -100 mV, recovery from inactivation was quite rapid: for example, a 200-ms interval between two successive 10-s jumps from -100 to 0 mV was sufficient to restore 64% of the peak current. However, when the holding potential was -50 mV, recovery from inactivation was markedly slower: note that the time-scale is 20-times slower for the curve corresponding to the -50 mV holding potential than for that corresponding to the -100 mV holding potential. The strong voltage-dependence of the kinetics of recovery from inactivation was observed in a total of four experiments where measurements were alternatively performed for -50 and -100 mV. Results were independent of the order with which the two holding potentials were applied. In these four experiments, the time for half-recovery was 115 ± 30 ms and 3.3 ± 1.1 s at -100 and -50 mV respectively. These findings required that the slow recovery from inactivation at -50 mV be taken into account in the programming of voltage protocols.

Fig. 1D gives the inactivation curve of I_{K1}; this curve was obtained by applying depolarizing jumps to a constant test potential (0 mV), successively from various holding potentials (-100 to -25 mV). In four similar experiments, half-inactivation was observed at -40 ± 3.5 mV.

We tested several classical potassium channel blockers in order to further characterize the potassium current of rat osteoblasts. As shown in Fig. 2A and B, both tetraethylammonium (TEA, 1 mM) and quinine (10 μM) reduced I_{K1} without markedly changing its inactivation kinetics. I_{K1} was reduced by $39 \pm 9\%$ (10) by TEA 1 mM and by $40 \pm 6\%$ (4) (mean $\pm \sigma_n(n)$) by quinine 10 μM. I_{K1} could be reduced by 85% by 10 mM TEA and completely blocked by 100–300 μM quinine (3 cells, not shown). As shown in Fig. 2C, 4-amino-pyridine (4-AP) 1 mM was also able to reduce the peak current activated by depolarization to 0 mV. However, this agent also slowed down the inactivation kinetics of I_{K1}. These effects were observed in 4 cells (peak-current reduction at 0 mV of $37 \pm 12\%$).

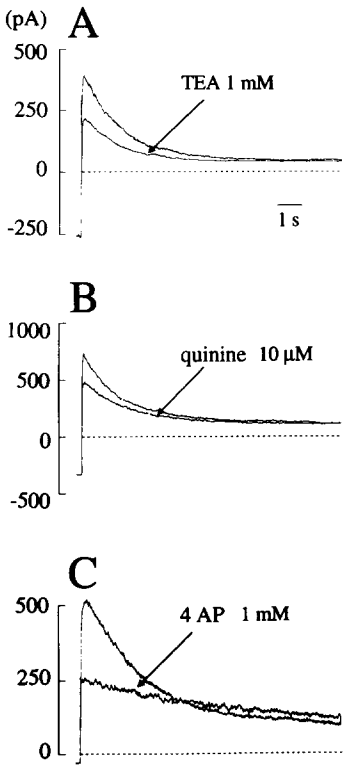

Fig. 2. Effects of TEA 1 mM (A), quinine 10 μM (B) and 4-AP 1 mM (C) on I_{K1} recorded during depolarizing jumps from -100 to 0 mV in three different cells. Note that 4-AP slows down the apparent inactivation of I_{K1}.

I_{K1} was not affected by either charybdotoxin (14 nM (4 cells) or 28 nM (2 cells)) or apamin (100 nM (2 cells)).

I_{K2}

I_{K1} remained stable while the same depolarizing jump was regularly applied after the beginning of the whole-cell dialysis. In addition to this current, we also observed in a few cells another outward current which spontaneously increased in amplitude with successive depolarizing jumps. This current appeared to be carried by K^+ ions, since it was outward at, and even below, the Cl^- equilibrium potential (0 mV) and reversed at the K^+ equilibrium potential (see Fig. 4).

Fig. 3A shows the spontaneous development of this current, measured at the end of 10-s depolarizing jumps from -50 to 0 mV. Current traces recorded during depolarizing jumps performed at the beginning of the whole-cell recording and after development of the spontaneously increasing current are superimposed in Fig. 3B, and the difference between these two traces is illustrated in Fig. 3C. The difference measured at 0 mV gives a current which was labeled I_{K2}. This current is clearly different from I_{K1}: instead of showing a slow inactivation during the 10-s depolarization to 0 mV, it shows on the contrary a slow activation. Such a slow activation could be noticed, even without subtraction

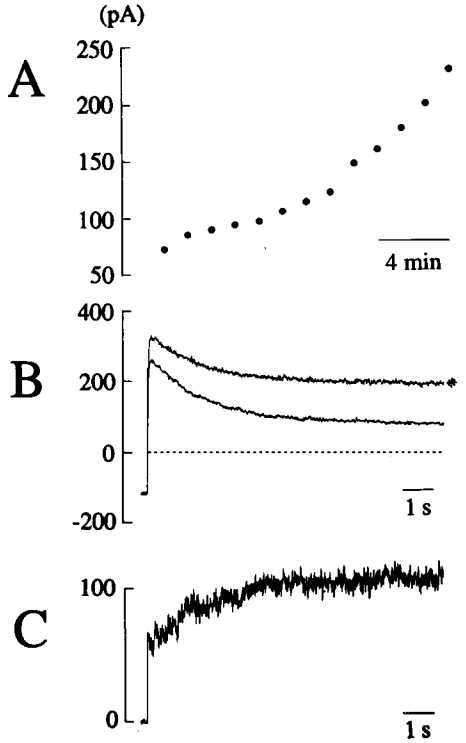

Fig. 3. Spontaneous increase of an outward current at 0 mV (I_{K2}) during successive depolarizing jumps from −50 mV. (A) Curves giving, for each successive depolarizing jump, the current value measured at the end of the depolarization. The first point was obtained 3 min after beginning of cell dialysis in the whole-cell configuration. (B) Current traces recorded during depolarizing jumps performed at the beginning of the recording (mean of the two first traces) and after development of I_{K2} (mean of the two last traces (*). (C) Difference between the two traces illustrated in B, showing the I_{K2} current. Contrary to I_{K1}, this current does not inactivate, but slowly activates during the 10-s depolarization to 0 mV.

of successive traces, by the observation that the spontaneous current increase was always larger at the end of the depolarizing jumps than at the peak of I_{K1} (35 cells).

The $I-V$ curve of I_{K2} was established by applying a series of depolarizing jumps from −100 mV to −80, −60, −40, −20, 0, +20 and +40 mV both at the beginning of the recording and after a spontaneous increase of I_{K2}, and by subtracting the first curve from the second one. In order to obtain a reliable $I-V$ curve for I_{K2}, we had to work under conditions in which the only current which had changed between the two curves was I_{K2}. As mentioned in Materials and Methods, at negative-membrane potentials most cells also showed an inward current which changed during cell dialysis. In the experiment of Fig. 4A, this inward current was blocked (not shown) by 1 mM Cd^{2+}, and the current level was very stable around the K^+ equilibrium potential (−84 mV), so that it was possible to get an $I-V$ curve for I_{K2}. Fig. 4Aa shows the evolution with successive jumps of the current measured at the end of each depolarization to 0 mV; periods of application of Cd^{2+} are indicated, and Cd^{2+} did not have much effect on I_{K2} (a result confirmed in two other experiments). The $I-V$ curve illustrated in Fig. 4Ab shows that, with the usual 5 mM K^+ external solution, I_{K2} reverses around the K^+ equilibrium potential (−84 mV), that this current can already be partially activated at negative membrane potentials, but shows outward rectification and slowly increases during strong depolarizing jumps.

The fact that I_{K2} is carried by K^+ ions was confirmed by the observation that the reversal potential of this current followed the K^+ equilibrium potential, when this potential was brought close to 0 mV, by using a high-K^+ external solution (Fig. 4B).

Whereas 1 mM TEA was able to reduce I_{K1} (see Fig. 2A above), it did not have any effect on I_{K2} (2 cells). This is shown in Fig. 5 which illustrates an experiment where depolarizing jumps from −100 mV to 0 mV were regularly applied every 20 s, and where I_{K2} developed. Both the peak outward current and the current measured after 10 s at 0 mV are plotted for successive depolarizing jumps. Whereas TEA reversibly reduced the peak current, it had little effect on the current measured after 10 s at 0 mV even after development of I_{K2}. These results, which were confirmed in another experiment, demonstrate that I_{K2} was not affected by TEA 1 mM, whereas I_{K1} was reduced. Increasing the dose of TEA to 20 mM induced a reduction of I_{K2} by about 32% (not shown).

I_{K2} was completely blocked by 100 μM quinine (1 cell) and was not affected neither by charybdotoxin 28 nM (2 cells) nor by apamin 100 nM (1 cell) (data not shown).

Effects of vitamin D-3 metabolites and cyclic GMP analogs on potassium currents

1,25-$(OH)_2D_3$ ($6 \cdot 10^{-9}$ M) induced a small increase in outward current during depolarizing jumps to various membrane potentials in 7 out of 12 cells. This outward current appeared to be carried by K^+ ions, since it was observed above the K^+ equilibrium potential (close to −84 mV), increased with increasing depolarization (see Fig. 6a), and could be detected at the Cl^- equilibrium potential (0 mV), or even below. This 1,25-$(OH)_2D_3$-induced increase of K^+ current was reversible and could be observed several times consecutively on a given cell. It was of small amplitude: 36 ± 21 pA ($n = 7$) (mean ± σ_n) at 0 mV (holding potential −100 mV). Fig. 6a gives the evolution with time of the current recorded at the end of depolarizing jumps from −100 mV to +30 mV, 0 mV or −20 mV, in an experiment in which the effects of $6 \cdot 10^{-9}$ M 1,25-$(OH)_2D_3$ were measured twice. Each test depolarizing jump was repeated every minute, in alternation with the two other depolarizing jumps. The reversible development of an outward current during the hormone

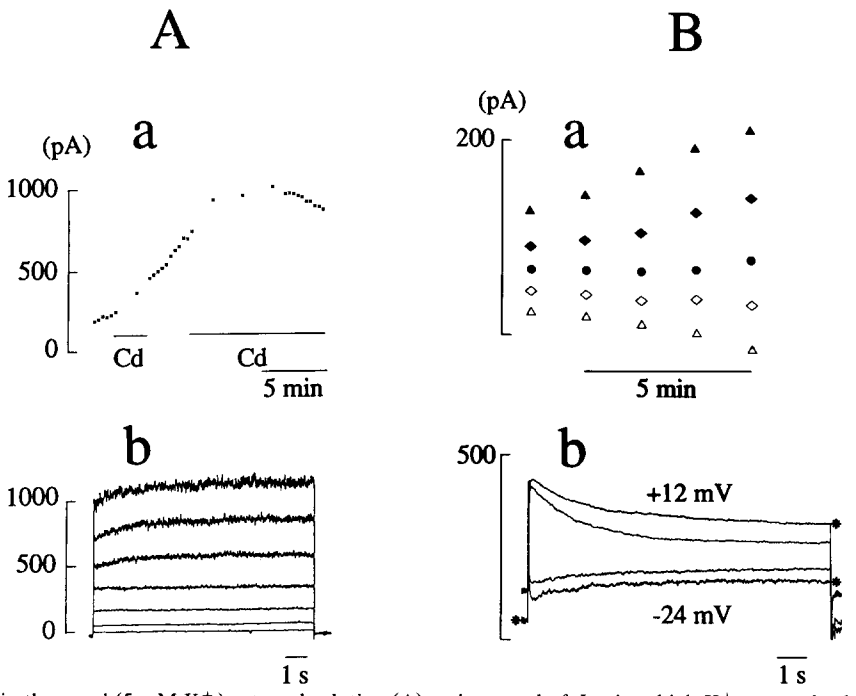

Fig. 4. $I-V$ curve of I_{K2} in the usual (5 mM K^+) external solution (A) and reversal of I_{K2} in a high-K^+ external solution (B). (A) Experiment performed with the usual external and internal solutions ($E_K = -84$ mV). (a) Current values measured at the end of 10 s depolarizing jumps from -100 mV to 0 mV for successive jumps. 10-s depolarizing jumps from -100 mV were applied every 20 s either to 0 mV (points illustrated) or to -80, -60, -40, -20, 0, $+20$ and $+40$ mV (interruptions). $CdCl_2$ 1 mM was applied (bars) in order to block an inward current which usually was observed at negative membrane potentials and changed in amplitude during cell dialysis. Comparison of the points obtained just before and just after the second addition of Cd^{2+} 1 mM shows that these ions do not affect I_{K2}. At the end of this experiment I_{K2} slightly decreased with successive jumps. This observation was also done in other cells, in the absence of Cd^{2+}: at the end of very long recordings, we could even observe complete suppression of I_{K2} in a few cells. (b) I_{K2} traces for depolarizing jumps from -100 mV to -80, -60, -40, -20, 0, $+20$ and $+40$ mV, obtained by subtracting the current traces recorded during the first series of voltage jumps from those recorded during the second series of voltage jumps (after the increase in I_{K2}). Note that I_{K2} is inward at -100 mV, close to zero at -80 mV, and shows outward rectification above E_K. (B) Experiment performed with the high-K^+ external solution and the low-Cl^- internal solution ($E_K = 1$ mV); the low-Cl^- internal solution was used in order to avoid the spontaneous changes of the Cd^{2+}-sensitive inward current. Series of 10-s depolarizing jumps from -100 mV to -24, -12, 0, $+12$ and $+24$ mV were regularly applied. (a) Current values measured for each series of depolarizing jumps, at the end of the depolarizations. Note that the current which developed during cell dialysis was outwardly directed at $+12$ and $+24$ mV, and was inwardly directed at -12 and -24 mV. The first series of voltage-jumps was applied 10 min after beginning of cell dialysis. (b) Current traces recorded during voltage jumps from -100 mV to -24 mV (lower traces) or $+12$ mV (upper traces), during the first series of voltage-jumps, and after development of I_{K2} (*). Note that a large inward I_{K2} current was also detected at -100 mV.

application is particularly clear at $+30$ mV, and is also detectable, although less pronounced, at 0 mV and -20 mV. Fig. 6b shows the current traces recorded during the 10 s depolarizing jumps from -100 to $+30$ mV, before and during 1,25-$(OH)_2D_3$. The 1,25-$(OH)_2D_3$-induced outward current is superimposed on the large basal K^+ current, which slowly inactivates during the 10 s depolarization. The 1,25-$(OH)_2D_3$-induced outward current has the same magnitude at the end of the depolarization and at the peak of the basal K^+ current; in other words, this hormone-induced current does not inactivate during the 10-s depolarization and therefore must be distinct from the basal I_{K1}.

1,25-$(OH)_2D_3$ had no clear effect on I_{K1}. It also had no effect on I_{K2}, in 6 cells in which this current developed, when applied at a concentration of 0.1–6 nM.

24,25-$(OH)_2D_3$, even at a very low concentration (10^{-11} M), could reproduce the effect of 1,25-$(OH)_2D_3$ in cells which did not show I_{K2} (data not shown). The amplitude of the response measured at 0 mV, with a holding potential of -100 mV, was 31 ± 3 pA (mean $\pm$ σ_n) in 3 cells which responded to 10^{-11} M 24,25-$(OH)_2D_3$, and was of the same order of magnitude (24 ± 13) in 6 cells in which the dose tested was $6 \cdot 10^{-9}$ M; another cell showed a much larger response to $6 \cdot 10^{-9}$ M 24,25-$(OH)_2D_3$ (170 pA).

But, unlike 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ reduced I_{K2}, in cells which showed this current. This is illustrated by Fig. 7 for two separate experiments (A and B). In the experiment of Fig. 7A, depolarizing jumps from -100 mV to 0 mV were regularly applied and two applications of 10^{-11} M 24,25-$(OH)_2D_3$ were performed. The increase of I_{K2} with successive depolariz-

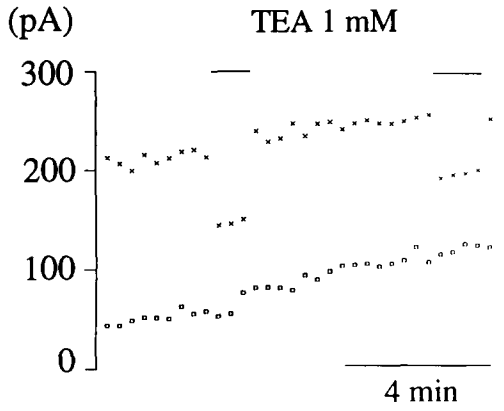

Fig. 5. Effect of 1 mM TEA on I_{K1} and I_{K2}. 10-s depolarizing jumps from −100 mV to 0 mV were regularly applied every 20 s, and the current is measured at 0 mV, for successive depolarizations, both at the peak of I_{K1} (×) and at the end of the depolarization (□). Two applications of 1 mM TEA were performed. Both reduced I_{K1}, as shown by the reduction of the peak current. The second TEA application, performed after a clear increase of I_{K2}, did not affect I_{K2}, as shown by the absence of effect of TEA at the end of the depolarization. The internal solution contained, in mM: 127 KCl, 14 NaCl, 1 $MgCl_2$, 0.1 EGTA, 3 Na_2ATP and 10 Hepes-NaOH (pH 7.0).

ing jumps is shown in Fig. 7Aa by plotting the current measured at the end of the successive jumps to 0 mV. 24,25-$(OH)_2D_3$ clearly reduced I_{K2}.

In the experiment of Fig. 7B, three successive applications of $6 \cdot 10^{-9}$ M 24,25-$(OH)_2D_3$ were performed while depolarizing jumps from −100 to −20, 0 and +30 mV were successively applied. Whereas the first hormone application, performed before development of I_{K2}, did not have much effect, the second and third hormone applications induced a strong reduction of I_{K2} which spontaneously developed during the beginning of the experiment (Fig. 7Ba). The current blocked by 24,25-$(OH)_2D_3$ was larger for larger depolarizations (Fig. 7Bb).

24,25-$(OH)_2D_3$ (10^{-11}–$6 \cdot 10^{-9}$ M) reduced I_{K2} in a total of 10 cells among 13 which showed this current.

In a few cases where the first application of 24,25-$(OH)_2D_3$ was performed before development of I_{K2}, we could observe on the same cell both effects of 24,25-$(OH)_2D_3$: that is, during the first hormone application, an increase in K^+ current, similar to that described in Fig. 6b, and, during the following hormone applications, made after development of I_{K2}, a decrease of I_{K2}. In cells showing no spontaneous development of I_{K2}, 24,25-$(OH)_2D_3$ never induced a decrease in outward current (nor an increase in inward current).

As vitamin D-3 metabolites may increase intracellular cyclic GMP in some cells, and since cyclic GMP analogues have been reported to increase the activity of a K^+ channel [32], we wondered whether extracellular application of permeable analogues of cyclic GMP could reproduce the K^+-current increase induced by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in cells which do not show I_{K2}. Surprisingly, we observed in such cells that 1 mM extracellular dibutyryl cyclic GMP slightly decreased the total K^+ current (Fig. 8). This effect was observed in 5 out of 6 cells. Another permeable analogue of cyclic GMP, 8-PCPT cGMP (8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate) (0.1–0.5 mM) had a similar effect (in 3 out of 4

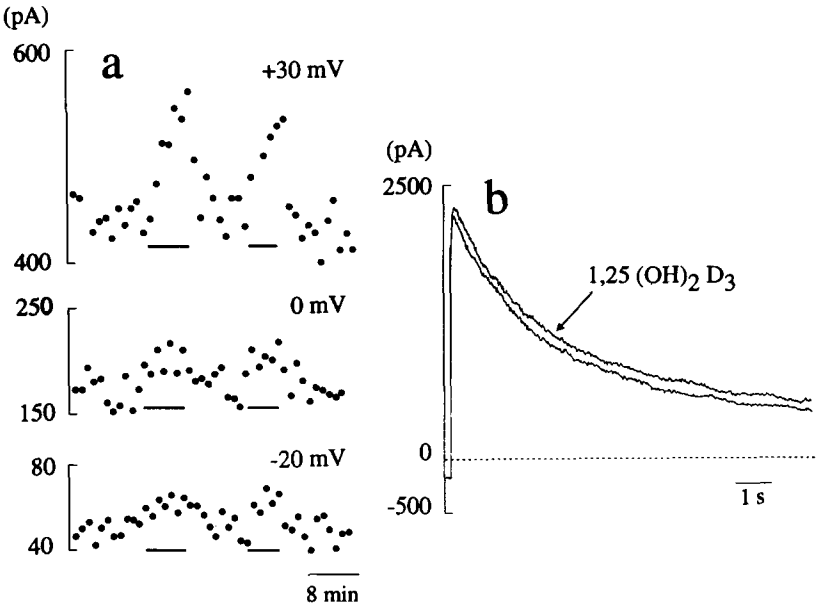

Fig. 6. 1,25-$(OH)_2D_3$ can activate a K^+ current of small amplitude. Data obtained from a cell where I_{K2} did not develop. 10-s depolarizing jumps from −100 mV to −20, 0 or +30 mV were alternatively applied (1-min interval between successive depolarizations to the same potential), and two applications of $6 \cdot 10^{-9}$ M 1,25-$(OH)_2D_3$ (indicated by the bars in a) were performed. (a) Successive current measurements at the end of each test depolarization. (b) Current traces recorded during depolarization from −100 to +30 mV before and during the second application of 1,25-$(OH)_2D_3$. Zero-current level is indicated by a dotted line.

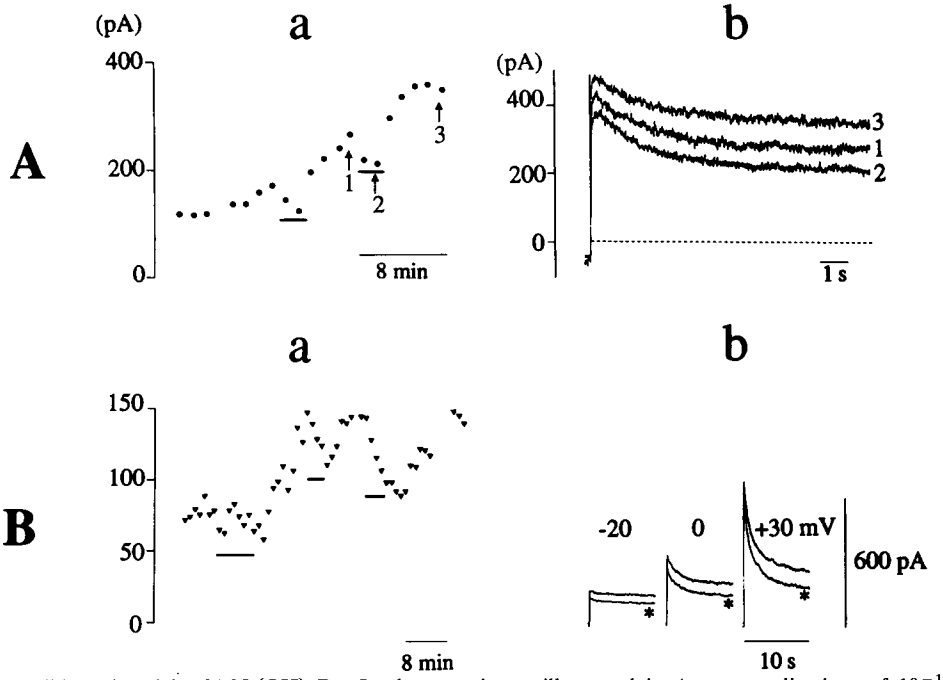

Fig. 7. I_{K2} can be reversibly reduced by 24,25-$(OH)_2D_3$. In the experiment illustrated in A, two applications of 10^{-11} M 24,25-$(OH)_2D_3$ (indicated by the bars in Fig. 7Aa) were successively performed. (Aa) Successive current measurements at the end of each depolarization from −100 mV to 0 mV. The first point was obtained 5 min after the beginning of whole-cell recording. Before the first 24,25-$(OH)_2D_3$ application, we observed a slow spontaneous increase in I_{K2}. This current is reversibly reduced by 24,25-$(OH)_2D_3$. Numbers indicate the points corresponding to the current traces illustrated in Ab. (Ab) Current traces recorded during depolarization from −100 mV to 0 mV before, during and after the second hormone application. In the experiment illustrated in B, 10 s depolarization jumps from −100 to −20, 0 and +30 mV were alternatively applied and three applications of $6 \cdot 10^{-9}$ M 24,25-$(OH)_2D_3$ (indicated by the bars in Fig. 7Ba) were successively performed. (Ba) Successive current measurements at the end of each depolarization to 0 mV. The first point was obtained 5 min after the beginning of whole-cell recording. (Bb) Mean of three successive current traces recorded during depolarization from −100 mV to −20, 0 and +30 mV before the third hormone application and at its maximal effect (*).

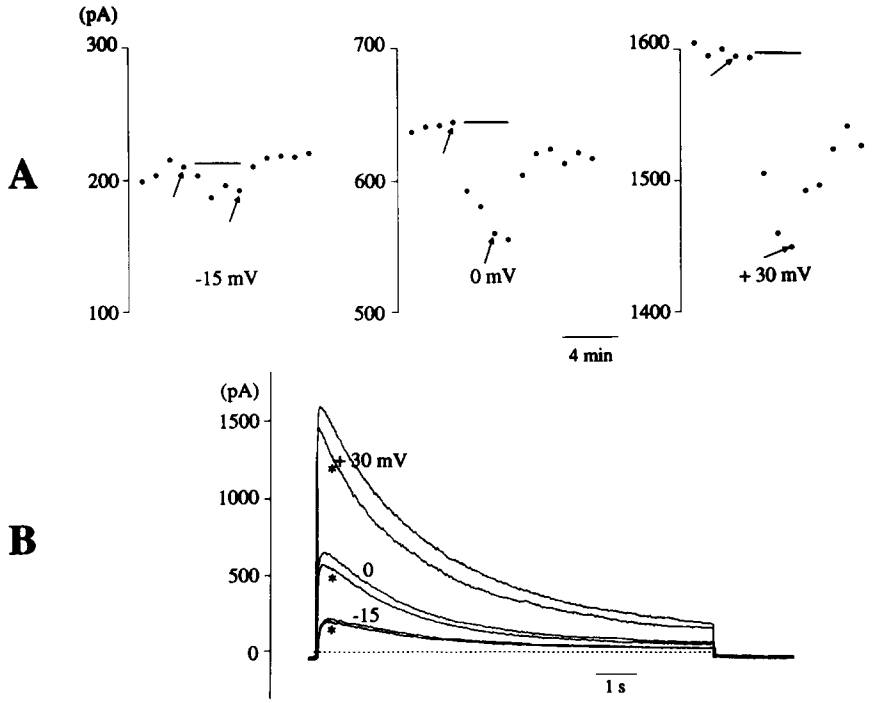

Fig. 8. K^+-current reduction induced by dibutyryl cyclic GMP. Depolarizing jumps from −100 mV to −15, 0 and +30 mV were alternatively applied (two successive points at a same test potential are separated by one minute). (A) Successive measurements of the peak outward current value at −15, 0 and +30 mV. Bars indicate the extracellular application of 1 mM dibutyryl cyclic GMP and arrows indicate the points corresponding to the traces illustrated in B. Note that the K^+-current reduction observed during the nucleotide application was reversible during wash and that it was not markedly voltage-dependent (peak current reduced by 10%, 12% and 9% at −15, 0 and +30 mV, respectively). (B) Superimposed current traces recorded before and during (*) the cyclic GMP analog application, during depolarizing jumps from −100 to −15, 0 and +30 mV.

cells). This effect was selective for cyclic GMP analogues, since 1 mM extracellular dibutyryl cyclic AMP did not reduce the K^+ current (5 cells).

Discussion

K^+ currents of rat osteoblasts

In most rat osteoblasts bathed in a physiological extracellular solution, we found that the main transmembrane ionic current recorded in the whole-cell configuration of the patch-clamp technique is a large voltage-dependent K^+ current, which is activated by depolarization, but which slowly inactivates when the depolarization is maintained for several seconds. This current, called I_{K1}, is TEA-, 4 AP- and quinine-sensitive but charybdotoxin- and apamin-insensitive. Its kinetics of recovery from inactivation is strongly voltage-sensitive, being about 20-times slower at -50 mV than at -100 mV. The pharmacological properties of this current are distinct from those of the delayed rectifier potassium current recently described in chondrocytes, which is inhibited by 10 nM charybdotoxin and unaffected by 10 mM TEA [33].

In a few cells, we also observed another K^+ current, called I_{K2}, which spontaneously increased in amplitude with time during the whole-cell recording. This current is clearly distinct from I_{K1}: instead of inactivating, it slowly increased in amplitude during a 10 s depolarization to 0 mV, so that it was always larger at the end of the depolarization than at the peak of I_{K1}; furthermore it was not affected by 1 mM TEA which reduces I_{K1} by about a half. The slow increase of I_{K2} with time is probably due to the slow dialysis of the intracellular medium by the pipette solution. This current does not seem to be a Ca^{2+}-activated K^+ current, since we could observe its development in cells which were dialysed with 10 mM BAPTA (1,2-bis (2-aminophenoxy)ethane-*N*,*N*N′,N′-tetraacetic acid), a strong Ca^{2+}-ion chelator (not shown).

Analogous effects of cell dialysis, facilitating the detection of a current, have been previously described in a few cases. We have shown in rat osteoblasts, that it is only in well-dialysed cells that stimulation of the adenylate cyclase (by either forskolin or parathyroid hormone) can activate a chloride current [27]. Two other groups have reported evidence for a cytosolic inhibitor of epithelial chloride channels [28,29], and the spontaneous activation of a Ca^{2+}-conductance during whole-cell recording of human leukemic T-cells has also been reported [34].

Effects of vitamin D-3 metabolites and cyclic GMP analogues

Vitamin D-3 metabolites were reported to increase the intracellular Ca^{2+} concentration of osteoblasts, and this effect seems to result partly from a release of intracellular Ca^{2+}, and partly from an entry of extracellular Ca^{2+} [4]. Since osteoblasts show both voltage-gated Ca^{2+} channels [26] and voltage-gated K^+ channels, it was of interest to know if vitamin D-3 metabolites might affect Ca^{2+} currents, not only directly [20], but also indirectly, via K^+-current modulations, which would induce membrane-potential changes and, therefore, Ca^{2+}-current changes.

In cells which did not show I_{K2}, the main K^+ current, I_{K1}, was not affected by vitamin D-3 metabolites. However, both 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ were able to induce a small K^+ current. This current did not inactivate during a 10-s depolarization from -100 to 0 mV, which distinguishes it from I_{K1}. The possibility that this hormone-induced K^+ current might be Ca^{2+}-activated remains to be tested.

Cyclic GMP analogues did not mimick vitamin D-3 metabolites in inducing a K^+-current increase, but on the contrary, induced a K^+-current decrease. This effect was selective for cyclic GMP analogues, since it could not be reproduced by cyclic AMP analogues. Another example of K^+-current blockade by cyclic GMP has been reported in a recent study of dissociated enterocytes [35].

In cells which showed I_{K2}, this current, which became the dominant current during cell dialysis, was strongly reduced by 24,25-$(OH)_2D_3$, even at very low doses (10^{-11} M). We never observed a reduction of I_{K2} by 1,25-$(OH)_2D_3$ even at much higher doses ($6 \cdot 10^{-9}$ M). It has been reported previously that 24,25-$(OH)_2D_3$ may exert unique action on bone formation [36,37]. However, this function is still controversial and all the recent reports of early effects of vitamin D-3 metabolites showed either no effect of 24,25-$(OH)_2D_3$ [3,5–7,11,38,39] or an effect which could also be induced by 1,25-$(OH)_2D_3$ [4,10]. Our results indicate that 24,25-$(OH)_2D_3$, which is a very abundant metabolite of vitamin D-3, may have effects which are not reproduced by 1,25-$(OH)_2D_3$.

If, in some cells, I_{K2} is already activated at the resting potential (which seems possible from the $I-V$ curve of this current), its blockade by 24,25-$(OH)_2D_3$ could induce a depolarization and thus, an increased Ca^{2+} entry through Ca^{2+}-channels activated by depolarization.

Our results confirm that 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ can induce effects within a few seconds (so-called 'early' effects). The rapidity of the responses that we observed and their induction by a very low concentration of 24,25-$(OH)_2D_3$ (10^{-11} M), suggest that they do not involve the specific 1,25-$(OH)_2D_3$ receptor.

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