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Cyclic AMP–Modulated Potassium Channels in Murine B Cells and Their Precursors

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A voltage-dependent potassium current (the delayed rectifier) has been found in murine B cells and their precursors with the whole-cell patch-clamp technique. The type of channel involved in the generation of this current appears to be present throughout all stages of pre-B-cell differentiation, since it is detected in pre-B cell lines infected with Abelson murine leukemia virus; these cell lines represent various phases of B-cell development. Thus, the presence of this channel is not obviously correlated with B-cell differentiation. Although blocked by Co^{2+}, the channel, or channels, does not appear to be activated by Ca^{2+} entry. It is, however, inactivated by high intracellular Ca^{2+} concentrations. In addition, elevation of intracellular adenosine 3′,5′-monophosphate induces at all potentials a rapid decrease in the peak potassium conductance and increased rates of activation and inactivation. Therefore, potassium channels can be physiologically modulated by second messengers in lymphocytes.

ALTHOUGH THEIR PHYSIOLOGICAL function has not been clarified, potassium channels have been detected in thymus-derived (T) cells (*1–4*) and their precursors (*5*) with the patch-clamp technique (*6*). These channels may play an important role in T-cell function, since the blockage of K^+ currents by exogenous specific drugs results in the loss of T-cell effector functions (*7*). Furthermore, K^+ channels can be modulated by mitogens (*1, 4, 5, 8*), which also regulate, in T cells, a recently described (*9*) inward current. None of these effects have been explained in terms of subcellular mechanisms.

In contrast, the only available data concerning ionic channels in bone marrow–derived (B) cells were obtained in hybridomas for which the presence of calcium channels was correlated with the ability to secrete immunoglobulins (Igs) (*10*). This scarcity of information about more usual forms of B and pre-B cells prompted us to determine the predominant type of channel in their membranes and the nature of intracellular signals influencing their behavior in order to gain clues about their possible functions.

Here we show (Fig. 1) outward voltage-dependent K^+ currents in a purified murine B cell treated for 72 hours in vitro with lipopolysaccharide (LPS), a potent activator of B lymphocytes (*11*). Depolarizing pulses from a holding potential of −80 mV elicited an outward current detectable at −40 mV, which increased in magnitude as the membrane potential was made more positive (Fig. 1A). Furthermore, as a result of prolonged depolarization this current inactivated and both activation and inactivation rates increased with increasing potential. Several observations indicate that this current is carried mainly by K^+ ions. (i) When the extracellular K^+ concentration was identical to that in the pipette, inward currents were elicited by voltage steps up to 0 mV, and above this value, outward currents were generated (Fig. 1B). (ii) Addition of tetraethylammonium chloride (TEA) (10 m*M*) or 4-aminopyridine (1 m*M*) to the bath blocked the channel almost completely. Furthermore, when intracellular K^+ ions were replaced by Cs^+ ions, no outward current was detectable. Thus, the type of K^+ channels we have described here is very similar to the one previously detected in a variety of cell types, including resting and activated T cells (*1–4*) and more recently in T cells in an early stage of differentiation (*5*).

We tested whether the presence of K^+ channels can be directly correlated with the degree of B-cell immunocompetency. Because the membrane of freshly collected splenocytes treated with antiserum to Thy-1 and complement (resting B cells) proved to be extremely fragile, we used cells at earlier stages of differentiation, immortalized with the Abelson murine leukemia virus (AMuLV), a retrovirus; substantial data on B-cell development have already been collected with these cells (*12, 13*). The ordered synthesis and expression of immunoglobulin molecules has long been used to characterize B lymphocytes at particular periods of their developmental pathway (*14*). Early stage pre-B cells do not produce Ig μ heavy chain (H) or κ light chain (L) molecules. As differentiation proceeds, H chains accumulate in the cytoplasm before L chains. We found that all AMuLV-infected pre-B cell lines tested display K^+ channels, which have properties that closely resemble those of the K^+ channel found in LPS-treated lymphocytes (blasts). As above, the threshold potential for activation of the current was around −40 mV in all cells studied; activation and inactivation displayed similar kinetics, although cell-to-cell variability may have prevented the detection of small differences between cell types. Along this line, the mean maximum K^+ conductance, which is slightly larger in the 18-81 clone (8.5 nS; SD, 4.1 nS), than for all the other cells taken together (5.1 nS; SD, 0.67 nS; $n = 5$) was the only noticeable discrepancy (Table 1). The reasons for such a discrepancy, which may be attributed to differences in cell size or in channel density, have not been investigated. However, these differences are, by far, much less than those observed in the course of T-cell cycling (*4*). These results suggest that the presence of K^+ channels in B cells is independent of their state of immunocompetency; thus, we have selected, for most of our further studies, the 18-81 line, which is, so far, the best characterized (*12, 15*). In this

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population, the current had the following characteristics: potential for half activation, −28 mV (SD, 4.8 mV; n = 14); for an 80-mV pulse from a holding potential of −80 mV, its half time to peak and half decay time were 3.3 msec (SD, 1.2 msec; n = 14) and 325 msec (SD, 74 msec; n = 11), respectively (calculated after 10 minutes of recording to allow equilibration; for conductances, see Table 1).

We investigated the relation between this potassium channel and calcium ions. High internal Ca^{2+} (10^{-5} to $10^{-6}M$) affects both the amplitude and the inactivation rate of K^+ currents in B cells (Fig. 2, A and B). When the solution in the pipette was buffered at low calcium ($10^{-8}M$) levels, the amplitude of the outward currents increased during the first 5 to 10 minutes of recording in 30 out of 33 cells (21%; SD, 27%); a small reduction of 9% (SD, 5%) was observed in the remaining three cells. A similar observation has been reported for T cells (*16*). When, on the other hand, the solution in the pipette contained $2 \times 10^{-6}M$ and $10^{-5}M$ calcium, an initial decrease of the currents of 22% (SD, 5.5%; n = 4) and of 27% (SD, 9%; n = 4), respectively, was observed during the first minutes of recording. This effect seems, however, less important than that reported for human T cells (*17*) as the mean maximum conductance for B cells equilibrated with solutions containing $2 \times 10^{-6}M$ calcium (6.1 nS; SD, 1.9 nS; n = 4) was not significantly lower than in the controls (8.5 nS; SD, 4.1 nS; n = 64). The effect of high intracellular calcium concentrations on the inactivation of the current was more striking. For an 80-mV pulse (from a holding potential of −80 mV) the half decay time was 244 msec (SD, 45 msec; n = 4) and 106 msec (SD, 75 msec; n = 4), in cells equilibrated with 2×10^{-6} and $10^{-5}M$ calcium, respectively, values that are obviously shorter than the 325 msec (SD, 74 msec; n = 11) in the control cells.

Table 1. Evidence that K^+ channels are present at all stages of pre-B cell differentiation. The different cell types are listed from top to bottom, according to their order of maturation, as defined by their cytoplasmic light and heavy chain expression. K^+ conductances (g_K) are expressed in nanosiemens. Characterization of cell lines: AMuVL lines were obtained from the femur of BALB/c and SJL mice, essentially as described in (*12*). All lines utilized for this report had been grown continuously in vitro for at least 5 months and had been obtained in our laboratories, except for 18-81, which was originally generated by Siden *et al.* (*12*). All clones, including those negative for both μ and κ, belong to the B-cell lineage, because they react with the monoclonal antibody BP1, which recognizes a marker selectively expressed by pre-B cells (*29*). The expression of cytoplasmic Ig chains was analyzed with rabbit polyclonal antibodies to IgM (directly fluoresceinated), and with a monoclonal rat antibody to mouse κ chain.

Cell line	Type	Cytoplasmic Ig chain expression		g_K (nS)		Sample size (n)
		μ	κ	Mean	SD	
BASP-1	Pre-B	−	−	5.5	1.0	3
Z-1-19	Pre-B	−	−	4.3	1.8	7
7-1-6	Pre-B	+	−	5.2	1.9	16
18-81	Pre-B	+	−	8.5	4.1	64
10-2	Pre-B	+	+	4.5	2.0	9
Blasts LPS	B	+	+	5.9	4.3	16

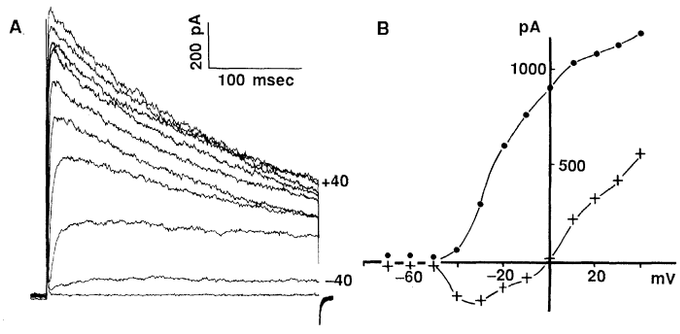

Fig. 1. Voltage-dependent outward currents in B lymphocytes. (**A**) A family of currents evoked in a B cell in the whole-cell configuration by depolarizing pulses given in 10-mV steps, from −50 mV to +40 mV, and delivered every 30 seconds to allow complete recovery from inactivation (normal solution; membrane potential held at −80 mV). (**B**) Relation of current to voltage obtained in different K^+ conditions. For each step potential, currents were measured at their peak amplitudes, and their values were derived after subtraction of the linear component of the leakage current, as explained below. (●) Same experiment as in (A). (+) Plot of the currents from another B cell, and in symmetrical K^+ conditions [same solutions as in (A) but with 150 mM K^+ as the main external cation]. Note that, in this case, the reversal potential was between 0 and −3 mV. Unpolished patch electrodes (≃ 1 Mohm) were pulled from soft glass and filled with a solution containing 150 mM KCl, 4 mM NaCl, 5 mM EGTA, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 mM Hepes buffer (*p*H 7.2). Just before recording (at room temperature), the cells were washed of incubation medium and bathed in 150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Hepes buffer (*p*H 7.2). Because the electrode resistances were low and the currents collected had slow activation kinetics, no series compensation was necessary, as checked by the identity between compensated and uncompensated recordings. Data analysis was performed with a PDP 11 MINC computer coupled to an analog-to-digital converter (INDEC). Voltage-dependent K^+ conductances were calculated as follows: the linear component of the leak was estimated with small hyperpolarizing or depolarizing pulses below threshold for activation of outward currents, and was subtracted, after scaling, from the measured peak current at each step potential (no inward rectifying current was noted). The derived value was then divided by the potential minus the reversal potential; the latter, which was estimated with the Nernst equation, equaled −91 mV in normal conditions.

The next step was related to the effect of external Ca^{2+}. A classical Ca^{2+} channel blocker such as Co^{2+} reversibly abolished the K^+ current (Fig. 2C). Comparable results were obtained when Cd^{2+} was substituted for Co^{2+}. However, activation of the K^+ channel is not a consequence of Ca^{2+} entry: currents recorded in cells bathed in a medium without Ca^{2+} displayed their usual kinetics (Fig. 2D). Therefore, the origin of the Co^{2+} effect remains to be elucidated. Because the maximum conductance is drastically reduced by the blocker (the remaining conductance in the presence of 10 mM Co^{2+} was 8 ± 9%, n = 4, of its control value), the blockage of the current is not solely due to a simple shift of the conductance to voltage curves, as would be expected if Co^{2+} was to change only membrane surface charges (*18*).

Furthermore, no voltage- and time-dependent inward current was detected in our experimental conditions in pre-B cells, in contrast to what was described in cultured myeloma cells (*19*). Blockage of the K^+ channels by 1 mM 4-aminopyridine in a medium containing high concentrations of Ca^{2+} did not reveal any inward current (Fig. 2E). We obtained the same results with TEA or with Cs^+-filled electrodes. Therefore, it appears that Ca^{2+} channel blockers can affect K^+ channels in B cells independently of Ca^{2+} fluxes, as already postulated for T lymphocytes (*2*). We cannot, however, completely rule out the existence of Ca^{2+} fluxes in intact cells, either through very labile Ca^{2+} channels or directly through K^+ channels, as is the case for the delayed rectifier current of the squid giant axon (*20*). Properties of voltage-insensitive channels, as described in T lymphocytes (*9*), have not been analyzed.

To further investigate the possible physiological role of this K^+ channel, we tested whether this current could be modulated by other intracellular signals involved in cell cycle control, such as adenosine 3′,5′-mono-

phosphate (cAMP) (*21, 22*). Increasing intracellular cAMP results in a marked decrease and, in some cases, in a complete disappearance of the outward current elicited by depolarizing pulses (Fig. 3). The addition of the adenylate cyclase activator forskolin (10 to 100 μM) (*23*) together with the phosphodiesterase inhibitor theophylline (1 mM) produced a rapid (less than 1 minute), dose-dependent reversible decrease of the peak voltage-dependent K^+ current at all potentials tested that was associated with an increase in the rate at which it inactivates (Fig. 3, A and B). In nine 18-81 cells, the currents were similarly reduced to 60% (SD, 11%) of their control values, and their half time of activation and of inactivation at 0 mV was shortened to 62% (SD, 22%) and to 70% (SD, 30%), respectively [control perfusions with dimethyl sulfoxide (DMSO) at 2% in Ringer solution or theophylline 1 mM, had no effect]. This overall reduction was not due to a shift of the voltage dependence of the channel, as shown by conductance-voltage plots (Fig. 3C), or to superimposition of an inward current, because blockade of the K^+ current by TEA after forskolin addition revealed no inward current. Similar results were obtained on LPS blasts.

We confirmed that forskolin does act via cAMP rather than in a nonspecific manner by (i) the use of pipettes free of both adenosine triphosphate (ATP) and guanosine triphosphate (GTP), in which case the effect of forskolin was transient (n = 3), and (ii) the addition of the adenylate cyclase antagonist adenosine (2 mM) in the pipette, which totally suppressed the effect of forskolin in four out of five cells.

GTP by itself altered the properties of the K^+ current in the first minutes of recording before the current reached a steady state; it caused a small initial decrease (19%; SD, 16%; n = 16) of the peak current elicited by successive pulses to 0 mV, a value to compare with the increase of 18% (SD, 26%; n = 33) for a solution without GTP (the presence of ATP alone made no difference). The significance of this effect is questionable, but it nevertheless may reflect either a regulation of the channel by a GTP-binding protein (*24*) or an indirect effect through the activation of a cascade involving cAMP production (*23*). We studied the reversibility of activation of adenylate cyclase effects by incubating the cells with forskolin plus theophylline before patch clamping. Replacement of the medium with normal Ringer solution resulted in a fast rise of the current amplitude. Finally, in confirmation of the above results, adding cAMP directly to the pipette solution led to the diminution of the outward current (of 25%; SD, 3.5%; n = 6), within 5 minutes after breaking into the cell, or to its nearly complete disappearance (n = 10) (Fig. 3D) when doses used were of 100 μM and 1 mM, respectively. These effects were associated with a progressive increase of the rate at which the current inactivated. This modulation of K^+ channels is analogous to that reported for action of cAMP-dependent kinases in other cell types (*25*).

The physiological role of K^+ channels in B as well as in T cells remains a matter of speculation, although a correlation between

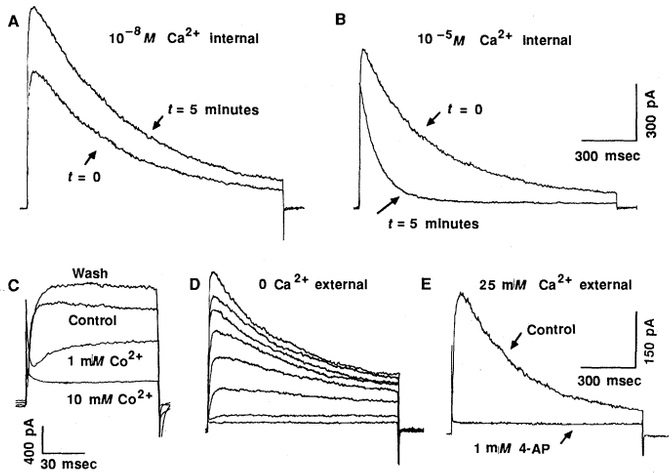

Fig. 2. Relation of the outward potassium current to internal and external calcium. (**A**) and (**B**) Representative K^+ currents elicited by 80-mV depolarizing pulses recorded just after breaking into the cell (t = 0) and 5 minutes later (t = 5 minutes); note the inverse evolution of the current in low (A, $10^{-8}M$) and high (B, $10^{-5}M$) intracellular Ca^{2+} concentrations. Extracellular solution as for Fig. 1A. Intracellular solution in the pipette: (A) as for Fig. 1, (B) as for Fig. 1, except 1.009 mM EGTA, 1.01 mM $CaCl_2$, $[Ca^{2+}]_{free} = 10^{-5}M$. For solution at $[Ca^{2+}]_{free} = 2 \times 10^{-6}$; 1.045 m$M$ EGTA, 1.002 mM $CaCl_2$ (see text for results). (**C**) K^+ currents evoked, in the indicated conditions, by 80-mV depolarizing pulses (same ionic conditions as for Fig. 1A). Note the reversible block produced by 10 mM Co^{2+} in the bath. (**D**) Currents recorded in absence of external Ca^{2+} from a cell bathed in a medium containing 0 mM $CaCl_2$, 0.5 mM EGTA, 2 mM $MgCl_2$, 4 mM KCl, and 150 mM NaCl. Depolarizing pulses in 10-mV steps from −50 to 20 mV. (**E**) Lack of detectable inward currents when K^+ currents were blocked by 1 mM 4-aminopyridine (4-AP) in high external Ca^{2+}, 80-mV pulses. External medium as in Fig. 1A except for NaCl (125 mM), and $CaCl_2$ (25 mM). Holding potential −80 mV for all cells. Cell line 18-81.

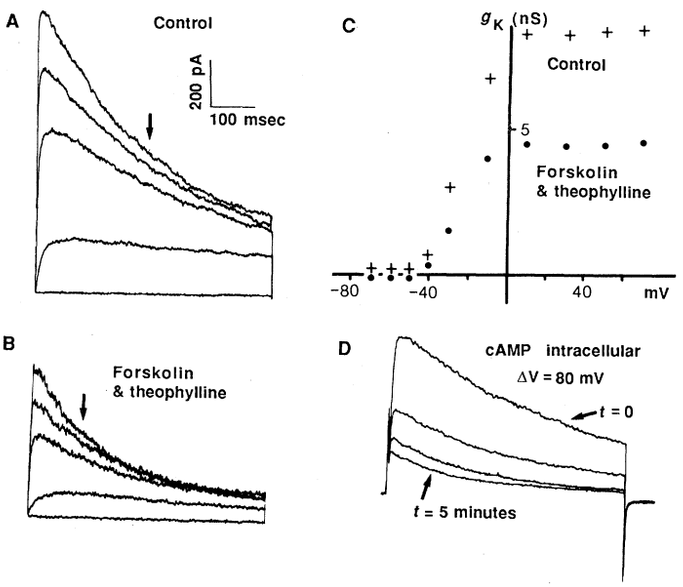

Fig. 3. Evidence that cAMP reduces potassium currents in B cells. Whole-cell recordings from the same pre-B lymphocyte before (**A**) and 3 minutes after (**B**) addition of forskolin (50 μM) plus theophylline (1 mM) in the bath. Step commands of 30, 50, 70, 90, and 110 mV were applied to the cell from a holding potential of −80 mV (records after subtraction of linear leakage current). Note that the peak amplitude of outward currents is reduced in presence of the drugs and that the rate of inactivation is enhanced, as indicated by the currents half decay time (arrows), which, for the uppermost trace, were 240 and 110 msec in (A) and (B), respectively. Forskolin (Calbiochem) was prepared from aliquots at 50 mM in DMSO. Theophylline was diluted in Ringer solution at 20 mM before final dilution. Solution in the pipette as for Fig. 1 plus GTP (100 μM) and ATP (5 mM). (**C**) Corresponding conductance to voltage relations (same cell as above) with a maximum conductance of 9.6 and 5.3 nS in the control (+) and after forskolin (●), respectively. (**D**) Effect of intracellular cAMP. Superimposed traces of currents elicited by pulses of the same amplitude shifting the potential of the cell from −80 mV to 0 mV, and obtained at times 0, 40 seconds, 3 minutes, and 5 minutes after breaking into the cell. Respective half decay times were, from top to bottom (in milliseconds), 280, 190, 110, and 80. Pipette solution as for Fig. 1 plus cAMP (1 mM), theophylline (1 mM) and ATP (5 mM). Pre-B cell lines 18-81 and 7-1-6 in (A) to (C), and (D), respectively. Note that the background noise in the presence of forskolin (B) is absent when cAMP is directly included in the pipette (D), thus suggesting that this effect is not cAMP mediated.

cell cycle progression induced by exogenous ligands and activity of K^+ channels has recently been demonstrated in the latter (*1, 4, 5, 8*). Similarly, the function, if any, of second messengers in the regulation of K^+ conductances in lymphocytes is unclear. Increase of free intracellular Ca^{2+} (*26*) as well as membrane depolarization (*27*) have been shown to occur during the early stages of B-cell activation, and the K^+ channel described here is a good candidate for mediating the last phenomenon. A physiological role for the modulating effects of cAMP is more difficult to define because, on one hand, the level of cAMP has been shown to increase during B-cell triggering (*22*) and, on the other hand, exogenous elevation of intracellular cAMP has been reported to inhibit both lymphocyte proliferation (*21, 22*) and the early events occurring during activation (*28*). Taken together, these data support the fact that cAMP exerts opposite effects during B-cell cycling (*22*). It remains to be established whether some of these different cAMP-modulated stages of cell cycling are related to activity of K^+ channels. The fact that K^+ currents are also modulated by cAMP in early pre-B cells suggests that at least this cyclic nucleotide has the potential to control K^+ channel activity during the developmental pathway of this lineage.

Because currents similar to those we have seen in B cells have been observed in T cells and their precursors (*5*), it is likely that these K^+ channels develop before the divergence of discrete B- and T-cell lineages, and it would be of importance to determine if T-cell channels are also controlled by cAMP.

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30. BALB/c spleen cell suspensions were first treated with ammonium chloride to lyse red cells and second with antiserum to Thy-1 and complement to eliminate T lymphocytes. The resultant cell population (more than 95% of the cells were Ig = positive) was cultured in RPMI-1640 supplemented with L-glutamine, specillin G2 (10^3 U/ml), streptomycin (10^3 U/ml) (Specia), 10% fetal calf serum and 2×10^{-5} 2-mercaptoethanol in the presence of 50 μg/ml of LPS from *Salmonella typhimurium* (Difco Laboratories) for 72 hours.

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Molecular Cloning of Complementary DNA Encoding the Avian Receptor for Vitamin D

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Vitamin D_3 receptors are intracellular proteins that mediate the nuclear action of the active metabolite 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. Two receptor-specific monoclonal antibodies were used to recover the complementary DNA (cDNA) of this regulatory protein from a chicken intestinal λgt11 cDNA expression library. The amino acid sequences that were deduced from this cDNA revealed a highly conserved cysteine-rich region that displayed homology with a domain characteristic of other steroid receptors and with the *gag-erbA* oncogene product of avian erythroblastosis virus. RNA selected via hybridization with this DNA sequence directed the cell-free synthesis of immunoprecipitable vitamin D_3 receptor. Northern blot analysis of polyadenylated RNA with these cDNA probes revealed two vitamin D receptor messenger RNAs (mRNAs) of 2.6 and 3.2 kilobases in receptor-containing chicken tissues and a major cross-hybridizing receptor mRNA species of 4.2 kilobases in mouse 3T6 fibroblasts. The 4.2-kilobase species was substantially increased by prior exposure of 3T6 cells to 1,25$(OH)_2D_3$. This cDNA represents perhaps the rarest mRNA cloned to date in eukaryotes, as well as the first receptor sequence described for an authentic vitamin.

VITAMIN D_3 UNDERGOES METABOLIC activation in liver and kidney to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active form principally responsible for the regulation of calcium and phosphorus homeostasis in higher vertebrates (*1*). The biologic response to 1,25$(OH)_2D_3$ in birds and mammals occurs through a complex series of events that are mediated by an intracellular receptor protein and that parallel those of the classical steroid hormones (*2*). Thus, the kinetics of induction of messenger RNAs (mRNAs) for the chicken and rat vitamin D_3–dependent calcium-binding proteins (*3, 4*), for example, are consistent with a genomic action of 1,25$(OH)_2D_3$, although control of the expression of these genes by a direct interaction of the 1,25$(OH)_2D_3$ receptor (VDR) with the nucleus has not been demonstrated. Apart from the classical roles assigned to 1,25$(OH)_2D_3$, it has been shown to participate in regulating such diverse functions as cellular proliferation and differentiation, secretion of polypeptide hormones, and induction of catabolic enzymes for the vitamin and modulating certain aspects of the immune system (*5*). These and certain other pleiotropic effects of the activated vitamin are under the control of VDR (*6*).

Chicken 1,25$(OH)_2D_3$ receptors (cVDR) are polypeptides of approximately 60 kD that selectively bind the active vitamin with high affinity and interact with nuclei and DNA both in vitro and in vivo (*7*). Nevertheless, limited structural and functional insight into this macromolecule has accumulated, primarily because its intracellular con-

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