The steroid hormone 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) rapidly modulates Ca²⁺ homeostasis in avian skeletal muscle cells by driving a complex signal transduction mechanism, which promotes Ca²⁺ release from inner stores and cation influx from the outside through both L-type and store-operated Ca²⁺ (SOC) channels. In the present work, we evaluated the involvement of calmodulin (CAM) in 1,25-(OH)₂D₃ regulation of SOC influx in chick skeletal muscle cells. Treatment with 10⁻⁹ M 1,25-(OH)₂D₃ in Ca²⁺-free medium resulted in a rapid but transient Ca²⁺ rise correlated with the sterol-induced inositol 1,4,5-trisphosphate (IP₃) production. The SOC influx stimulated by the hormone was insensitive to both CAM antagonists (fluphenazine, trifluperoxazine, chlorpromazine, compound 48/80) and the CAM-dependent protein kinase II (CAMKII) inhibitor KN-62 when added after the sterol-dependent Ca²⁺ transient, but it was completely abolished when added prior to the IP₃-induced mobilization of Ca²⁺ from endogenous stores. Moreover, in cells microinjected with antisense oligonucleotides directed against the CAM mRNA the sterol-stimulated SOC influx was reduced up to 60% respect to uninjected cells. The present results suggest that the 1,25-(OH)₂D₃-induced (IP₃-mediated) cytosolic Ca²⁺ transient is required for CAM activation which in turn activates SOC influx in a mechanism that seems to include CAMKII.

The steroid hormone 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) modulates calcium homeostasis in skeletal muscle cells by both a classical genomic action, which elicits control of gene expression (1, 2), and a non-genomic mechanism, implying direct membrane effects of the hormone (2–4) mediated by diverse signaling systems. In chick skeletal muscle cells, the steroid rapidly modulates Ca²⁺ influx by G protein-mediated activation of both phospholipase C (5, 6) and adenyl cyclase (7), leading to activation of PKC and PKA (5, 7–9), release of Ca²⁺ from inner stores (10), and activation of voltage-dependent Ca²⁺ channels (VDCC) from the L-type (11). We recently examined the action of 1,25-(OH)₂D₃ on cytosolic Ca²⁺ ([Ca²⁺]ᵢ) levels in these cells (10, 12). The cytosolic Ca²⁺ response to the hormone involves an initial rapid sterol-induced Ca²⁺ mobilization from IP₃/thapsigargin-sensitive stores followed by cation influx from the extracellular milieu, accounting for a sustained Ca²⁺ phase. This Ca²⁺ influx was shown to be contributed not only by the well established L-type VDCC-mediated Ca²⁺ entry but also by a store-operated Ca²⁺ entry (SOCE) pathway, thus introducing a novel aspect into the mechanism of 1,25-(OH)₂D₃-induced Ca²⁺ influx across the plasma membrane of muscle cells. The information obtained points to a role for both PKC and tyrosine kinase activities, but not the cyclic AMP/PKA cascade, in the regulation of the sterol-dependent SOCE pathway.

In previous studies it was shown that the rapid changes in Ca²⁺ influx induced by 1,25-(OH)₂D₃ in both intact soleus muscle and cultured myoblasts are paralleled by an increase in the amounts of calmodulin (CAM) bound to membranes at the expense of a decrease in its cytosolic concentration without any change in total CAM cellular content (13, 14). There is a diversity of cellular responses controlled by Ca²⁺-dependent pathways utilizing CAM as the signaling mediator (15), the activation and/or modulation of which occurs either through direct interaction with target proteins or via CAM-dependent activation of regulatory proteins (16). Although a plethora of signaling cascades and putative mechanisms have been related to SOCE regulation (17, 18), mobilization and/or depletion of Ca²⁺ from endogenous stores seems to be an almost ineludible step in SOC channel activation. In the present work we investigated whether CAM was involved in the mechanism by which 1,25-(OH)₂D₃ stimulates SOC entry in chick skeletal muscle cells. The results suggest that the 1,25-(OH)₂D₃-induced cytosolic Ca²⁺ transient is required for CAM activation, which in turn activates SOC influx in a mechanism that might include the CAM-dependent protein kinase II (CAMKII).

**EXPERIMENTAL PROCEDURES**

**Chemicals—**1α,25-(OH)₂D₃ was kindly provided by Dr. Heinrich Bachmann (Hoffmann-La Roche). Dulbecco’s modified Eagle’s medium (DME), fetal bovine serum (FBS), Fura-2/AM, pluronic F-127, calmidazolium, chlorpromazine, compound 48/80, trifluperoxazine, and fluphenazine were from Sigma. KN-62 was from Calbiochem. Lipofectin was from Life Technologies, Inc. Monoclonal (mouse) Anti-CAM antibody (IgG₁) was from Affinity Bioreagents Inc. (Golden, CO). The ECL chemiluminescence detection kit was from Amersham Pharmacia Biotech. All other reagents were of analytical grade.

**Cell Culture—**Chick skeletal muscle cells were isolated from the breast muscle of 13-day-old chick embryos (Gallus gallus) and cultured at 37 °C in DME plus 10% FBS as described previously (11). Cells were allowed to grow until confluence (4–6 days after plating) before use, by which time all of the myoblasts become differentiated into myotubes.
expressing both the biochemical and morphological features of adult skeletal muscle fibers (19).

Intracellular Calcium Measurements—For intracellular Ca\textsuperscript{2+} measurements, cells grown onto glass coverslips were loaded with 4 \mu m Fura-2/AM in buffer A (containing in mM) 138 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 5 glucose, 10 Heps (pH 7.35, 1.5 CaCl\textsubscript{2} plus 0.1% bovine serum albumin 0.05% and 0.012% pluronic) for 45 min at room temperature. Unloaded dye was washed out, and cells were stored in buffer B (buffer A without bovine serum albumin, Fura-2/AM, and pluronic F-127) in the dark at room temperature for at least 40 min prior to use. Cells were then mounted on the stage of an inverted fluorescence microscope (Diaphot 200, Nikon Inc., Melville, NY) equipped with a 40 x NA objective lens. The excitation wavelength was rapidly (5.5 Hz) switched over 340 and 390 nm, employing a dual excitation monochromator (DMX1100, Spectronics Inc., Urbana, IL) from an SLM-Aminco 8100 spectrofluorometer (Spectronics Inc.) connected to the epifluorescence port of the microscope through an optic fiber. Emitted fluorescence was reflected by a 400 nm dichroic mirror and filtered through a 510 nm band pass filter. Ratios from short and long wavelength signals were obtained (r = 340/390), and for each single cell or cell group measured (each recording represents the response of at least 200–300 cells analyzed), calibration of cytosolic Ca\textsuperscript{2+} signal was accomplished in situ at the end of each experiment as described previously (12). A determination of changes in cytosolic Ca\textsuperscript{2+} was achieved by overlaying the cells with 500 \mu l of a nominally Ca\textsuperscript{2+}-free buffer B plus 0.5 mM EGTA.

Microinjection of Oligonucleotides—Cells were seeded (10,000/mm\textsuperscript{2}) on coverslips imprinted with squares for localization of injected cells. Oligodeoxynucleotides (ODN) with phosphorothioate linkages throughout the entire molecule were synthesized by the DNAgency (Malvern, PA). Injection of ODN was performed by a manual injection system (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The injection solution contained 10–15 \mu M ODN in sterile deionized water; 10–30 fl were injected with commercially microcapillaries (Femtotips, Eppen- dorf). Injection pressure was 60 hextops and the injection time 1 s. After injection, cells were cultured in DME containing 1% FBS for 48 h before fluorimetric measurements. The following ODN sequences were used: anti-CAM, 5′-ATACGCCATGTTGCTCGGCG-3′ (antisense on the 49–68 nucleotide sequence from Gallus gallus CAM mRNA); sense-CAM, 5′-CTGGAGCGACCATGCGTAT-3′; scrambled, 5′-ATC CTTAGGTCGGTACTT-3′.

Cell Transfection—Transfection with ODNs using Lipofectin was performed according to manufacturer’s instructions. ODNs were incubated with Lipofectin in DME for 15 min at room temperature. Plates of subconfluent cells were washed to remove serum before the addition of DME-Lipofectin mixtures, and incubation was performed for 12 h at 37 °C. The ODN solution was removed, DME was added, and the plates were placed into a metabolic incubator for an additional period of 36 h. Control treatments included DME only and Lipofectin only. Dose- and time-response curves for Lipofectin and ODN were optimized previously (not shown).

Immunoblotting of CAM—Cell homogenization, SDS-polyacrylamide gel electrophoresis (15% gels) protein fractionation, transfer to polyvinylidene difluoride membranes, and immunostaining of blots with mouse anti-CAM antibody and peroxidase-conjugated anti-mouse IgG, as well as visualization and digitalization of immunoreactive bands, were as described previously (20, 21).

Statistical Analysis—The statistical significance of data was evaluated using Student’s t test (22), and probability values below 0.05 (p < 0.05) were considered significant.

RESULTS AND DISCUSSION

As shown previously (10, 12), stimulation of chick skeletal muscle cells with 10\textsuperscript{-9} M 1,25-(OH)\textsubscript{2}D\textsubscript{3} in Ca\textsuperscript{2+}-free medium results in a rapid (30 s) but transient rise in cytosolic Ca\textsuperscript{2+} (Fig. 1) entirely because of mobilization from IP\textsubscript{3}-sensitive stores. Ca\textsuperscript{2+} re-admission to the bath, once the IP\textsubscript{3}-transient occurred, causes a fast and substantial entry of Ca\textsuperscript{2+} (about 2-fold over basal) from the outside. This procedure has been shown to reveal the existence of a Ca\textsuperscript{2+} influx pathway that becomes activated by the depletion of endogenous Ca\textsuperscript{2+} stores (10, 23, 24). Although blocking of VDCC by either nifedipine or verapamil does not affect the Ca\textsuperscript{2+} influx that normally follows Ca\textsuperscript{2+} re-addition, in the following experiments SOC influx was recorded in the presence of 2 \mu M nifedipine and 5 \mu M verapamil to ensure functional isolation of a SOC entry pathway (see Ref. 12).

To determine whether CAM was involved in the mechanism by which 1,25-(OH)\textsubscript{2}D\textsubscript{3} stimulates SOC entry, the effects of different CAM antagonists were assayed. Fig. 2A shows the effect of a classical CAM antagonist, fluphenazine (50 \mu M), on the sterol-dependent SOC entry. Application of the inhibitor 2 min prior to hormone-induced discharge of intracellular Ca\textsuperscript{2+} stores completely prevented Ca\textsuperscript{2+} influx after cation re-admission to the bath. In addition to fluphenazine, a series of CAM antagonists as well as Ken-62, a specific inhibitor of the CAMKII (25), were tested for their ability to block 1,25-(OH)\textsubscript{2}D\textsubscript{3}-dependent SOC influx. As for fluphenazine, the addition of trifluoperazine, chlorpromazine, compound 48/80, or the CAMKII inhibitor before the 1,25-(OH)\textsubscript{2}D\textsubscript{3}-induced cytosolic Ca\textsuperscript{2+} transient reduced the subsequent Ca\textsuperscript{2+} entry through the SOCE pathway by more than 80% (Fig. 2B). However, the addition of either the CAM antagonists or Ken-62 after the sterol-induced Ca\textsuperscript{2+} transient, but 2 min prior to Ca\textsuperscript{2+} re-admission to the bath, had no effect on the SOCE pathway (Fig. 3). The possibility arises that the lack of inhibition by all of the inhibitors tested could be due to a significantly shorter preincubation time (2 min) with these compounds than in the situation of an application prior to hormone exposure (8-min delay from antagonist addition to Ca\textsuperscript{2+} re-admission; Fig. 2); however, a 10-min delay interval between fluphenazine or Ken-62 addition after the Ca\textsuperscript{2+} transient and Ca\textsuperscript{2+} re-addition did not affect the sterol-dependent SOC influx (data not shown). Activation of CAM requires an elevation in cytosolic Ca\textsuperscript{2+}, which in turn binds to the protein activating it. Once activated, the Ca\textsuperscript{2+}-complexed CAM becomes insensitive to CAM antagonists. Moreover, Ken-62 inhibits the kinase activity of CAMKII by interacting with the CAM binding site of the enzyme but does not affect the CAM-independent activity of already active enzyme (25). The present results are consistent with the concept that the 1,25-(OH)\textsubscript{2}D\textsubscript{3}-induced IP\textsubscript{3}-mediated Ca\textsuperscript{2+} transient is a critical step in the activation of CAM and thus in the mechanism by which CAM activates SOC influx. This mechanism seems to involve CAM-mediated activation of CAMKII, in line with the notion that phosphorylation events are crucial in the modulation of the SOC channel function (17). As we observed essentially the same effects with the CAM antagonists as with the CAMKII inhibitor, we cannot exclude a role of CAM itself in the regulatory process. Although it has been shown by others that some CAM antagonists could directly inhibit both VDCC and SOC channels (26, 27), the experiments shown in Figs. 2 and 3 allow us to exclude a direct inhibition of the CAM inhibitors with the channel itself, which would be observed regardless at which point of the assay the drug is added to the bath. However, interaction of these antagonists with components of the signaling mechanism regulating SOC influx other than CAM cannot be ruled out from these data. Antisense technology allows selective inhibition of the expression of one particular protein involved in signal transduction and to inves-
FIG. 2. 1,25-(OH)$_2$D$_3$-induced SOC influx in skeletal muscle cells is abolished by pretreatment with either CAM antagonists or a CAMKII inhibitor. A, cells were incubated in the presence of fluphenazine (Ffz, 50 μM) for 2 min and then stimulated with $10^{-9}$ M 1,25-(OH)$_2$D$_3$ in Ca$^{2+}$-free medium. The right arrow indicates Ca$^{2+}$ (1.5 mM) re-addition to the bath. Shown is a time-trace recording representative of at least 257 cells. B, cells were incubated for 2 min in the presence of fluphenazine (Ffz, 50 μM), trifluoperazine (Tfz, 50 μM), chlorpromazine (Cpz, 40 μM), compound 48/80 (15 μM), or KN-62 (10 μM) and then stimulated with $10^{-8}$ M 1,25-(OH)$_2$D$_3$ in Ca$^{2+}$-free medium. As in panel A, after the Ca$^{2+}$ transient occurred, Ca$^{2+}$ (1.5 mM) was re-added to the bath. At least 290 cells were analyzed for each condition. Results are expressed as the amplitude of [Ca$^{2+}$], change in percent after re-addition of Ca$^{2+}$ compared with controls (hormone alone, 100%).

FIG. 3. Addition of either the CAM antagonists or a CAMKII inhibitor after the 1,25-(OH)$_2$D$_3$-induced cytosolic Ca$^{2+}$ transient does not affect the sterol-dependent SOC influx in skeletal muscle cells. A, cells were stimulated with $10^{-9}$ M 1,25-(OH)$_2$D$_3$ in Ca$^{2+}$-free medium, and the sterol-dependent Ca$^{2+}$ transient was monitored until completion. Then fluphenazine (Ffz, 50 μM) was added, and 2 min later Ca$^{2+}$ (1.5 mM) was re-admitted to the medium. Shown is a time-trace recording representative of 260 cells analyzed. B, cells were stimulated with the steroid as in panel A; after the Ca$^{2+}$ transient, fluphenazine (50 μM), trifluoperazine (50 μM), chlorpromazine (40 μM), compound 48/80 (15 μM), KN-62 (10 μM), or vehicle (isopropanol < 0.01%) was added, and 2 min later Ca$^{2+}$ (1.5 mM) was re-added to the medium. At least 250 cells were analyzed for each condition. Results are expressed as in Fig. 2B.

Tigate how its knock-out affects a determined cell function (28). This technique, used in combination with fluorimetric detection of hormone-induced changes in [Ca$^{2+}$], has proven to be a powerful tool for identifying signaling cascades involved in VDCC regulation (29, 30). To determine the extent to which complete abolition of 1,25-(OH)$_2$D$_3$-stimulated SOC influx by CAM antagonists reflects an absolute requirement of the sterol-dependent SOCE pathway for CAM activity, we inhibited the expression of CAM by intranuclear microinjection of an antisense ODN against the CAM mRNA from G. gallus. In cells microinjected with the anti-CAM ODN, the SOC influx induced by 1,25-(OH)$_2$D$_3$ was reduced by as much as 60% in respect to the control (Fig. 4). Neither the sense ODN nor the scrambled ODN was able to modify the magnitude of the sterol-dependent SOC entry. In skeletal muscle cells the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$/Mg$^{2+}$ ATPase inhibitor thapsigargin induces a SOC influx highly similar to that dependent upon 1,25-(OH)$_2$D$_3$ stimulation but driven by signaling mechanisms different from those triggered by the steroid (12). It is important to note that the injection of cells with the anti-CAM ODN did not affect the SOC channel itself, as the SOCE pathway induced by thapsigargin remained intact (Fig. 4B). Moreover, this finding suggests that 1,25-(OH)$_2$D$_3$-regulated CAMKII activity modulates proteins other than those forming the channel itself but still closely associated, regulatory ones. The immunoblotting of CAM from lysates of cells transfected with the ODNs (Fig. 4C) proved the ability of the antisense ODN to suppress the expression of the targeted CAM protein. The remaining SOC influx observed in CAM-depleted cells suggests that additional signaling mechanisms are involved in the regulation of 1,25-(OH)$_2$D$_3$-induced SOC influx in muscle cells. In fact, previous data from our laboratory indicate that sterol-dependent activation of both PKC and tyrosine kinases (TKs) strongly contributes to the modulation of SOCE (12). To address the possibility that PKC or TKs could be involved in the residual SOC influx observed in CAM-depleted cells, we assayed SOC influx sensitivity to either PKC or TK inhibitors. Bisindolylmaleimide I (50 nM) and calphostin C (100 nM), two highly specific PKC inhibitors, added into the incubation medium 2 min prior to Ca$^{2+}$ re-admission almost completely suppressed (up to 98%) the residual SOCE in CAM-depleted cells, whereas under the same conditions, a 60% reduction was produced by the TK antagonists genistein (50 μM) and herbimycin (10 μM). This finding agrees with evidence indicating that both TK- and PKC-mediated protein phosphorylation are involved in the modulation of SOCE (see Ref. 17 and references therein). Although not directly addressed here, it seems likely that TK-mediated regulation of the SOC influx induced by 1,25-(OH)$_2$D$_3$ occurs in a PKC-dependent fashion, as no remnant SOC influx could be observed in the presence of PKC inhibitors. In addition, it has been observed recently that PKC inhibition blocks 1,25-(OH)$_2$D$_3$ stimulation of the TK cascade (2). Moreover, the residual SOCE measured in CAM-depleted cells was completely insensitive to fluphenazine, trifluoperazine, or KN-62.

S. Morelli, R. Boland, and A. R. de Boland, submitted for publication.
Calmodulin Role in 1,25-(OH)₂D₃-induced SOC Entry

Thus ruling out the possibility that residual CAM could be mediating such Ca²⁺ entry.

At present we lack a defined molecular mechanism responsible for signaling between 1,25-(OH)₂D₃-induced Ca²⁺ store depletion and SOC influx activation (see Fig. 5). The nature of the non-genomic actions of 1,25-(OH)₂D₃ has led to the proposition that a putative membrane receptor for the hormone is involved in the activation of signaling systems in muscle cells as postulated for this and other steroids in different cell types (2), but the existence of such a novel receptor has not been conclusively demonstrated. Other lines of evidence have alternatively pointed to a role of the classical vitamin D receptor (VDR) itself in mediating some of the rapid effects of the hormone (31, 32). However, compounds CB1093 and GS1500, two agonists with high affinity for VDR (31, 32), failed to induce any increase in Ca²⁺ mobilization in response to 1,25-(OH)₂D₃ (33). Although these results suggest that a receptor other than VDR mediates sterol actions on the SOCE pathway, the application of antisense technology to deplete CAM or a scrambled (S) ODN against the mRNA of CAM were homogenized 48 h later, and the CAM content of the lysates was evaluated.

Efforts using this experimental approach are currently under way in our laboratory. The general scenario of SOC influx triggering and regulation appears at present complex, involving Ca²⁺ influx-soluble factors, G proteins, phosphatases, and several kinases (reviewed in Refs. 17 and 34). The carboxy-terminal regions of transient receptor potential (TRP) and TRP-like channel proteins from Drosophila melanogaster photoreceptor cells, which function as Ca²⁺-permeable channels mainly regulated by store depletion, contain, respectively, one and two CAM-binding sites (35). Although several TRP and TRP-like homologues from vertebrate tissues have been cloned and characterized as candidates for mediating SOC influx in animal cells (see Ref. 36 and references therein), none of them exhibits CAM-binding motifs as their invertebrate counterparts do. Moreover, evidence on the functional roles of CAM on either vertebrate or invertebrate SOC channel function remains scarce. In this context, the present work not only enhances our knowledge on the role of CAM in the non-genomic mechanism by which 1,25-(OH)₂D₃ activates and modulates SOC entry in chick muscle cells but also contributes to the understanding of the function of CAM and its target protein CAMKII in animal cell SOC influx regulation.

REFERENCES

1. Boland, R. (1986) Endocr. Rev. 7, 434–448
2. Boland, R., de Boland, A. R., Marinissen, M., Santillan, G., Vazquez, G. & Zanello, S. (1995) Mol. Cell. Endocrinol. 114, 1–8
3. de Boland, A. R. & Boland, R. (1987) Endocrinology 120, 1585–1586
4. de Boland, A. R. & Boland, R. (1994) Cell. Signal. 6, 717–724
5. Morelli, S., de Boland, A. R. & Boland, R. (1995) Biochem. J. 309, 675–679
6. Morelli, S., Boland, R. & de Boland, A. R. (1996) Mol. Cell. Endocrinol. 122, 207–211
7. Vazquez, G. & de Boland, A. R. (1996) Biochim. Biophys. Acta 1310, 157–162
8. Vazquez, G., Boland, R. & de Boland, A. R. (1995) Biochim. Biophys. Acta 1289, 91–97
9. Vazquez, G., de Boland, A. R. & Boland, R. (1997) Biochem. Biophys. Res. Commun. 234, 125–128
10. Vazquez, G., de Boland, A. R. & Boland, R. (1997) Biochem. Biophys. Res. Commun. 239, 562–565
11. Vazquez, G. & de Boland, A. R. (1993) Biochem. Mol. Biol. Int. 31, 677–684
12. Vazquez, G., de Boland, A. R. & Boland, R. (1998) J. Biol. Chem. 273, 33954–33960
13. de Boland, A. R., Massheimer, V. & Fernández, L. (1989) Calcif. Tissue Int. 43, 370–375
14. Fernández, L., Massheimer, V. & de Boland, A. R. (1999) Calcif. Tissue Int. 47, 314–319
15. James, P., Vorherr, T. & Carafoli, E. (1995) Trends Biochem. Sci. 20, 38–42
Calmodulin Role in 1,25-(OH)_{2}D_{3}-induced SOC Entry

16. Braun, A. P. & Schulman, H. (1995) *Annu. Rev. Physiol.* **57**, 417–445
17. Montell, C. (1997) *Mol. Pharmacol.* **52**, 755–763
18. Putney, J. W. & Bird, G. S. (1990) *Endocr. Rev.* **14**, 610–631
19. Capiati, D. A., Limbozzi, F., Tellez-Inon, M. T. & Boland, R. (1999) *J. Cell. Biochem.* **74**, 292–3000
20. Capiati, D., Tellez-Inon, M. T. & Boland, R. (1999) *Mol. Cell. Endocrinol.* **153**, 39–45
21. Laemmli, U. K. (1970) *Nature* **227**, 680–685
22. Snedecor, G. & Cochrane, W. (1967) *Statistical Methods*, Iowa State University Press, Ames, IA
23. Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, R., Stefani, E. & Birnbaumer, L. (1996) *Cell* **85**, 661–671
24. Xu, X., Kitamura, K., Lau, K., Muzzlem, S. & Miller, T. (1995) *J. Biol. Chem.* **270**, 29169–29175
25. Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. & Hidaka, H. (1990) *J. Biol. Chem.* **265**, 4315–4320
26. Li, G., Hidaka, H. & Wollheim, C. B. (1992) *Mol. Pharmacol.* **42**, 489–498
27. Cao, Y. & Chatton, J.-Y. (1998) *FEBS Lett.* **424**, 33–36
28. Albert, P. R. & Morris, S. J. (1994) *Trends Pharmacol. Sci.* **15**, 250–254
29. Kleuss, C., Hescheler, J., Ewele, C., Rosenthal, W., Schultz, G. & Wittig, B. (1991) *Nature* **353**, 43–48
30. Kalkbrenner, F., Derguwar, V. E., Schenker, M., Brendel, S., Zobel, A., Hescheler, J., Wittig, B. & Schultz, G. (1995) *EMBO J.* **14**, 4728–4737
31. Kim, Y. S., MacDonald, P. N., Dedhar, S. & Hruska, K. A. (1996) *Endocrinology* **137**, 3649–3658
32. Barsony, J., McCoy, W., Renyi, I. & Liberman, M. E. (1994) in *Vitamin D, a Pluripotent Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications* (Norman, A. W., Bouillon, R., and Thomasset, M., eds) pp. 345–346, Walter de Gruyter, Berlin
33. Vazquez, G., Selles, J., de Boland, A. R. & Boland, R. (1999) *Br. J. Pharmacol.* **126**, 1815–1823
34. Berridge, M. J. (1995) *Biochem. J.* **312**, 1–11
35. Phillips, A. M., Bull, A. & Kelly, L. E. (1992) *Neuron* **8**, 631–642
36. Vannier, B., Peyton, M., Boulay, G., Brown, D., Qin, N., Jiang, M., Zhu, X. & Birnbaumer, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 2060–2064