The effects of protein kinase C stimulation on free cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) were studied in Fura 2-loaded UMR-106 cells. Stimulation of the protein kinase C with the tumor-promoting phorbol esters 12-O-tetradecanoylphorbol 13-acetate (TPA) and phorbol 12,13-diacetate or 1-oleoyl-2-acetylgllycerol was followed by an increase in [Ca\textsuperscript{2+}]\textsubscript{i}. The protein kinase C-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} has a lag period, the duration of which was dependent on the stimulant and medium Ca\textsuperscript{2+} concentrations. With 10 nM TPA, the rise in [Ca\textsuperscript{2+}]\textsubscript{i} peaked within 1.5 min, after which [Ca\textsuperscript{2+}]\textsubscript{i} returned partially toward baseline. The increase in [Ca\textsuperscript{2+}]\textsubscript{i} was absolutely dependent on the presence of medium Ca\textsuperscript{2+} and was inhibited by the Ca\textsuperscript{2+} channel blockers nifedipine and verapamil. Cell stimulation also results in Ca\textsuperscript{2+} release from intracellular pool(s) which appears to be mediated by a Ca\textsuperscript{2+}-dependent Ca\textsuperscript{2+} release mechanism. The reduction in [Ca\textsuperscript{2+}]\textsubscript{i} was due to channel inactivation. Pretreatment of the cells with 1 mM TPA, 2 units/ml parathyroid hormone (PTH), or 10 \muM forskolin blocked the effect of 2 \muM TPA on [Ca\textsuperscript{2+}]\textsubscript{i}. TPA and PTH were more potent inhibitors than was forskolin. The properties of this channel are compared to the cAMP-independent PTH-stimulated Ca\textsuperscript{2+} channel present in these cells.

The role of protein kinase C in cell stimulation has been documented in many cell systems. Activation of protein kinase C is mediated by diacylglycerol production in the cytosol (1). Tumor-promoting phorbol esters mimic the effects of endogenous diacylglycerol in activating protein kinase C (2). Phorbol esters provide an important tool for studying the role of protein kinase C in several cellular functions, including their effect on agonist-mediated changes in free cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}).

In secretory cells such as gastric peptic and pancreatic acinar cells, activation of protein kinase C can substitute for the slow phase of secretion induced by hormones (3, 4). In these systems, activation of protein kinase C does not involve changes in [Ca\textsuperscript{2+}]\textsubscript{i}, and has no effect on hormone-dependent increases in [Ca\textsuperscript{2+}]\textsubscript{i}. In other cells such as basophils, neutrophils, and platelets (5–7), activation of protein kinase C prior to the agonist stimulation leads to an inhibition of agonist-mediated changes in [Ca\textsuperscript{2+}]\textsubscript{i}. Activation of protein kinase C also inhibits a Ca\textsuperscript{2+} channel in a neuroblastoma cell line (8).

Recent reports show that although activation of protein kinase C has no effect on \textsuperscript{4}Ca accumulation by the adrenal medulla, protein kinase C activation facilitates \textsuperscript{4}Ca accumulation and subsequent catecholamine secretion induced by nicotine and high potassium in the extracellular medium (9). Stimulation of the adrenal medulla by nicotine and potassium involves activation of plasma membrane Ca\textsuperscript{2+} channels (9, 10). On the other hand, stimulation of protein kinase C enhances the Ca\textsuperscript{2+} current in aplysia neurons (11). Thus, there is evidence that protein kinase C activation can modulate the activity of Ca\textsuperscript{2+} channels, and the effect of protein kinase C on calcium channel activity (inhibitory or stimulatory) appears to be cell-specific.

Data on the role of protein kinase C stimulation on bone function and activity is meager. Tashjian (12) found that phorbol esters stimulated bone resorption in neonatal mouse calvaria. However, the effect of phorbol esters and protein kinase C activation in specific bone cell types has not been characterized. It is possible that the calcitropic hormones PTH and 1,25-dihydroxycholecalciferol can stimulate protein kinase C activity. The effect of these hormones on bone remodeling has been studied in organ culture, in isolated cells, and in bone-derived tumor cell lines (13–16). They have been found to stimulate bone resorption (17, 18), suppress collagen synthesis (18, 19), and at higher doses, inhibit proliferation of osteoblast-like cells while at lower doses stimulate proliferation of these cells (20).

All these actions of the calcitropic hormones were attributed to changes in cellular cAMP (19). However, recently we reported that PTH stimulation of the osteosarcoma cell line UMR-106 also induced an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (21). Using the same cell line, it was reported that PTH stimulated the production of phosphatidylinositol (22, 23), and, therefore, it is likely that PTH also stimulates the protein kinase C through the production of diacylglycerol from phosphatidylinositol phosphates (24). The PTH-dependent increase in [Ca\textsuperscript{2+}]\textsubscript{i} was initially mediated by activation of a cAMP-independent plasma membrane Ca\textsuperscript{2+} channel (21). However, the mechanism of activation of this channel by PTH remains unclear. We now report the presence of a protein kinase C-activated plasma membrane Ca\textsuperscript{2+} channel in the UMR-106 osteosarcoma cell line. The properties of this channel appear...
to be similar to those of the cAMP-independent PTH-activated Ca²⁺ channel.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol esters and OAG were obtained from Sigma. Bovine PTH(1-34) ("-" units/mg) was obtained from Beckman Instruments. The acetoxymethylester of Fura 2 (Fura 2/AM) was obtained from Molecular Probes (Eugene, OR). Forskolin was obtained from Behring Diagnostic. All other reagents used were of the highest grade available.

**Culture Conditions**—UMR-106 cells were used between passages 7 and 9. Cells were seeded at a density of 0.5-1.0 X 10⁶ cells/cm² in 25-cm² area flasks and grown at 37 °C in a humidified 95% air, 5% CO₂ atmosphere in Ham's F12: Dulbecco's modified Eagle's media (1:1) supplemented with 14.3 mM NaHCO₃, 1.2 mM L-glutamine, 7% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin. The cells reached confluence within 7-8 days and were used on day 8 or 9 of growth.

**Determination of [Ca²⁺]**—Measurements of free cytosolic calcium concentration were made by incorporating the calcium-sensitive fluorescent probe, Fura 2, into UMR-106 cells which had been released from tissue culture flasks by rapid treatment with trypsin (0.5%) and EDTA (0.2%) in Dulbecco's phosphate-buffered saline. Trypsin/EDTA treatment took place for approximately 3 min at 37 °C followed by a rapid 25-fold dilution with medium A (composition in mM: NaCl 140; KCl 5; MgCl₂ 1; CaCl₂ 1.5; HEPES/Tris 10, pH 7.4; glucose 6; bovine serum albumin, Fraction V 0.1%) and washing twice by centrifugation at 600 x g for 5 min at 25 °C. Cells were resuspended in medium A and incubated with 2 μM of Fura 2/AM in a shaking water bath at 37 °C for 30 min. After completion of Fura 2 loading, the cells were washed in medium A and resuspended in this medium. Approximately one million cells/2 ml were used in each set of experiments. Fluorescence was measured in a Perkin-Elmer 650-40 fluorescence spectrophotometer at excitation and emission wavelengths of 340 and 500 nm, respectively, with slits of 3 and 12 nm, respectively.

**Calibration of the Fura 2 Signal**—The Fura 2 signal was performed by methods similar to those described for the calibration of another fluorescent Ca²⁺ probe, Quin 2 (25). Briefly, medium Ca²⁺ was adjusted to 2 nm, and the cells were lysed with digitonin (25 pg/ml) to obtain the maximum fluorescence. Next, 10 mM EGTA and sufficient NaOH to elevate the pH to 8.5 were added to obtain the minimum fluorescence. The dissociation constant for Ca²⁺-Fura 2 was assumed to be 220 nM (26).

**RESULTS**

**Fig. 1** shows the effects of TPA, PDA, OAG, and 4α-phorbol on [Ca²⁺], of Fura 2-loaded UMR-106 cells in the presence of extracellular Ca²⁺. The maximal dose of TPA (2 μM) increased [Ca²⁺], from a resting level of 88 nM (124 ± 14, n = 30) to 474 nM (422 ± 14, n = 30). Subsequent to the addition of TPA, there was a characteristic lag period of 5-15 s. This lag period was followed by a rise in [Ca²⁺], which peaked within 1.5 min. The decrease in [Ca²⁺]; during the recovery phase is characterized by a reduction of [Ca²⁺], to about 250 nM, but [Ca²⁺], did not return to the nonstimulated basal level. PDA and the membrane-permeant analogue of diacylglycerol, OAG, were also able to increase [Ca²⁺], but they were less effective compared to TPA. Although maximal doses of PDA and OAG were used, it is noted that with these stimulants, the lag period was longer, the rate of increase in [Ca²⁺], was slower, and the maximum increase in [Ca²⁺], was lower. Fig. 1 also shows that the nontumor promotor 4α-phorbol at a concentration of 25 μM had no effect on [Ca²⁺].

**Fig. 2** shows the dose-response curves for TPA and OAG with respect to the increase in [Ca²⁺]. The maximal increase in [Ca²⁺], was found at 2 μM TPA and about 500 μM OAG. Fifty percent of the maximal increase in [Ca²⁺], was obtained with 0.5 μM TPA and 10 μM OAG. When increasing concentrations of TPA or OAG were used, the lag period decreased, the rate of rise of [Ca²⁺], increased, and the [Ca²⁺], rose to higher levels. Fig. 2 shows the dose-response curve for the peak increase in [Ca²⁺]. Similar dose-response curves were obtained when the lag period or the rate of increase in [Ca²⁺], was plotted against the concentrations of the agonist (not shown).

The dependence of the TPA-mediated increase in [Ca²⁺], on medium Ca²⁺ concentration is shown in Fig. 3 (left panel). Addition of 2 μM TPA to cells suspended in medium containing 2.5 mM Ca²⁺ increased [Ca²⁺], from 157 to 340 μM with a
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Fig. 3. Extracellular Ca\textsuperscript{2+} dependency of TPA-mediated rise in [Ca\textsuperscript{2+}]. Left panel, the experimental protocol for measurements of [Ca\textsuperscript{2+}], was the same as in Fig. 1 except that the cells were added to medium containing 2.5 mM CaCl\textsubscript{2}, 0.5 mM CaCl\textsubscript{2}, or 100 \mu M EGTA in the absence of added CaCl\textsubscript{2}. The cells were incubated in EGTA-containing medium for 1.5 min prior to stimulation. Right panel, UMR-106 cells loaded with Fura 2 were added to 2 ml of medium A containing the indicated CaCl\textsubscript{2} concentrations. After stabilization of the fluorescence signal (about 1 min), the cells were stimulated with 2 \mu M TPA, and [Ca\textsuperscript{2+}], was measured during the following 5 min. The increase in [Ca\textsuperscript{2+}], over resting level measured at 5 mM medium CaCl\textsubscript{2} (421 ± 27 (n = 5)) was taken as control 100%. At each medium CaCl\textsubscript{2} concentration, the increase in [Ca\textsuperscript{2+}], over the corresponding basal level was measured and calculated as the percent increase compared to the increase in [Ca\textsuperscript{2+}], observed with 5 mM CaCl\textsubscript{2}. The data shown are the mean ± S.D. of three to five separate experiments using three subpassages.

Fig. 4. Effect of Ca\textsuperscript{2+} channel blockers on TPA-mediated increase in [Ca\textsuperscript{2+}]. Cells were incubated in medium containing 2 mM CaCl\textsubscript{2} and stimulated with 2 \mu M TPA. The increase in [Ca\textsuperscript{2+}], over basal level was taken as 100%. The cells were also exposed to the indicated concentrations of nicardipine (■), verapamil (○), or diltiazem (▲). The effective concentrations of the blockers reduced the resting [Ca\textsuperscript{2+}]. Therefore, the cells were incubated with the blockers for 0.5-1.5 min until the signal stabilized and prior to stimulation with TPA. The increase in [Ca\textsuperscript{2+}], over the corresponding new basal level was measured and calculated as percent of the increase in [Ca\textsuperscript{2+}], in the absence of the blockers which was 312 ± 10 (n = 3). The data are the mean ± S.D. of three separate experiments using two subpassages.

Nicardipine was found to be the most potent blocker with a $K_i$ of approximately 0.6 \mu M and maximum inhibition at 10 \mu M. Verapamil at a concentration of 15 \mu M was required for 56% inhibition, while 25 \mu M diltiazem inhibited only 15% of the signal.

The experiments shown in Figs. 3 and 4 suggest that the TPA-mediated increase in [Ca\textsuperscript{2+}], is due only to Ca\textsuperscript{2+} entry into the cell through a plasma membrane Ca\textsuperscript{2+} channel. To test whether Ca\textsuperscript{2+} release from intracellular stores also contributes to the TPA signal, we measured the size of these stores with the Ca\textsuperscript{2+} ionophore, ionomycin (Fig. 5, top panel). Addition of 1.7 mM alkaline EGTA to cells suspended in medium containing 1.5 mM Ca\textsuperscript{2+} was followed by reduction in [Ca\textsuperscript{2+}], from 156 to 116 nM. Ionomycin at a concentration of 0.2 \mu M transiently increased [Ca\textsuperscript{2+}], to 930 nM. However, recovery of [Ca\textsuperscript{2+}], toward base-line levels was rapid, and [Ca\textsuperscript{2+}], stabilized at about 90 nM within 15 min. Addition of 2 \mu M ionomycin had no further effect on [Ca\textsuperscript{2+}]. Furthermore, 2 \mu M ionomycin increased [Ca\textsuperscript{2+}], to the same level as 0.2 \mu M, indicating that 0.2 \mu M ionomycin was sufficient to release all of the Ca\textsuperscript{2+} from intracellular stores. Fig. 5 (top panel) also shows that 0.2 \mu M ionomycin added to cells suspended in medium containing 0.5 mM CaCl\textsubscript{2} elevated [Ca\textsuperscript{2+}], to only 930 nM, and the cells were able to reduce [Ca\textsuperscript{2+}], back to 197 nM within 2.5 min of incubation at 37 °C. Subsequent addition of ionomycin at a concentration of 2 \mu M saturated the cytosol with Ca\textsuperscript{2+}. This experiment indicates that the increased Ca\textsuperscript{2+} permeability induced by 0.2 \mu M ionomycin was sufficient to deplete all of the Ca\textsuperscript{2+} from intracellular stores. Therefore, ionomycin can be used to measure the effect of TPA on intracellular Ca\textsuperscript{2+} content in the presence and absence of medium Ca\textsuperscript{2+}. Stimulation of the cells with TPA increased Ca\textsuperscript{2+} from 131 to 446 nM (Fig. 5, bottom panel). Removal of medium Ca\textsuperscript{2+} with 1.7 mM EGTA was followed by a rapid reduction in [Ca\textsuperscript{2+}], back to about 130 nM. Subsequent addition of 0.2 \mu M ionomycin increased [Ca\textsuperscript{2+}], to only 623 nM. When a second sample of cells was incubated with TPA in Ca\textsuperscript{2+}-free medium, ionomycin increased Ca\textsuperscript{2+} to 1111 nM. The same effect of ionomycin was found when the cells were incubated in Ca\textsuperscript{2+}-free medium and no TPA. This experiment shows that TPA stimulation of the cells reduced the amount
of Ca\(^{2+}\) in the intracellular stores only if Ca\(^{2+}\) was present in the medium during TPA stimulation. In five similar experiments, stimulation of cells suspended in Ca\(^{2+}\)-containing medium with TPA reduced the size of the ionophore-mobilizable intracellular Ca\(^{2+}\) stores by 39.2 ± 6.1%.

As shown in Fig. 1, the TPA-mediated increase in [Ca\(^{2+}\)]\(_{i}\) persisted for at least 15 min after a partial return toward baseline [Ca\(^{2+}\)]. The partial reduction in [Ca\(^{2+}\)]; could be due to partial inactivation of the plasma membrane Ca\(^{2+}\) channel or a stimulation of plasma membrane Ca\(^{2+}\) extrusion mechanisms. To explore the possibility of channel inactivation, we tested the effect of nicardipine or the removal of medium Ca\(^{2+}\) on [Ca\(^{2+}\)]; at different times after TPA stimulation (Fig. 6). Addition of nicardipine or EGTA to the medium before TPA stimulation reduced [Ca\(^{2+}\)]; from about 97 to 67 nM. However, when nicardipine or EGTA was added 1.5, 5, or 10 min after TPA stimulation, the cells were not able to reduce [Ca\(^{2+}\)]; back to the prestimulated level (Fig. 6, bottom). The progressive loss of nicardipine or EGTA sensitivity with time supports the possibility of channel inactivation with time. Additional evidence that the channel inactivated with time is given in Fig. 7. Stimulation of the cells with TPA was followed by a typical transient increase in [Ca\(^{2+}\)];. When medium Ca\(^{2+}\) was increased from 2 to 5 mM, only a small increase in [Ca\(^{2+}\)]; was observed (Fig.

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**Fig. 5.** Effect of stimulation by TPA on Ca\(^{2+}\) content of internal pool(s). Top panel, cells were added to 2 ml of solution A containing 1.5 (A, B) or 0.5 (C) mM CaCl\(_2\). Then 1.7 mM EGTA (pH 8.4) was added to the medium. Alkaline EGTA was used to prevent any change in medium pH which was maintained at 7.4 after EGTA addition. This concentration of EGTA reduced medium Ca\(^{2+}\) concentration to about 40 nM. The cells were then exposed to the indicated concentrations of the Ca\(^{2+}\) ionophore. The experiment shown is representative of 12 others. Ionomycin was dissolved and stored in dimethyl sulfoxide. The potency of ionomycin changed with time. Thus, the potency of ionomycin was determined before use in subsequent experiments. Bottom panel, cells were added to medium A containing 1.5 mM CaCl\(_2\); A, the cells were stimulated with 2 \(\mu\)M TPA for 1.5 min prior to addition of 1.7 mM alkaline EGTA. After an additional 2 min of incubation, the cells were exposed to 0.2 \(\mu\)M ionomycin. B, cells suspended in medium containing 1.5 mM CaCl\(_2\); were exposed to 1.7 mM EGTA. 1 min later the cells were stimulated with 2 \(\mu\)M TPA for 2 min before addition of 0.2 \(\mu\)M ionomycin. C, 1.7 mM EGTA was added to cells suspended in medium containing 1.5 mM CaCl\(_2\). After 2 min of incubation the cells were exposed to 0.2 \(\mu\)M ionomycin. This experiment is representative of four others.

**Fig. 6.** The effect of nicardipine (NiCa) and EGTA on [Ca\(^{2+}\)]; before and after stimulation with TPA. UMR-106 cells were suspended in medium containing 1.5 mM CaCl\(_2\) and stimulated with 2 \(\mu\)M TPA. As indicated, before or at different times after the stimulation with TPA, the cells were exposed to 10 \(\mu\)M nicardipine (A) or 1.7 mM alkaline EGTA (B). Each figure was constructed by superimposing five separate experiments with the same cells. This experiment is representative of two other similar experiments. The effect of nicardipine and EGTA on resting [Ca\(^{2+}\)]; and on [Ca\(^{2+}\)]; after 10 min of stimulation with TPA is representative of at least 14 similar observations.

**Fig. 7.** Effect of added CaCl\(_2\) on [Ca\(^{2+}\)]; of cells prestimulated with TPA. Cells were added to medium containing 2 mM CaCl\(_2\) (A) or 0.1 mM EGTA (B) and were then stimulated with 2 \(\mu\)M TPA. Where indicated, additional 3 mM (A) or 2.1 mM (B) CaCl\(_2\) was added to the medium. This experiment is representative of three others.
Increasing medium Ca\textsuperscript{2+} concentration from 100 nM to 2.0 mM 4 min after TPA stimulation increased [Ca\textsuperscript{2+}] from 86 to 142 nM (Fig. 7, bottom). The observation that [Ca\textsuperscript{2+}] did not rapidly increase after the addition of Ca\textsuperscript{2+} to cells pretreated with TPA again supports the possibility that the TPA-activated channel was not active by 4-5 min after TPA stimulation.

It is possible that the stimulation of protein kinase C which activates a plasma membrane Ca\textsuperscript{2+} channel can also regulate that same channel and be responsible for its inactivation. To test this hypothesis, we examined the effect of prior TPA preincubation on the TPA-activated Ca\textsuperscript{2+} channel. Stimulation of the cells with 2 \mu M TPA increased Ca\textsuperscript{2+} from 122 to 541 nM in this experiment (Fig. 8, top). Preincubation of the cells with 1 nM TPA had only a small effect on [Ca\textsuperscript{2+}]. This concentration of TPA decreased the ability of 2 \mu M TPA to increase [Ca\textsuperscript{2+}] in a time-dependent manner (Fig. 8, bottom).

The properties of the TPA-activated Ca\textsuperscript{2+} channel described so far resemble those of the PTH-activated cAMP-independent Ca\textsuperscript{2+} channel (21). To test whether this feedback mechanism is a property of TPA alone, we examined the effect of forskolin and PTH on the TPA-mediated increase in [Ca\textsuperscript{2+}]. Fig. 9 (top panel) shows that pretreatment of the cells with forskolin, which elevates cAMP in these cells (21), also inhibited the effect of TPA. As we showed previously, elevation of cellular cAMP by itself activates a plasma membrane Ca\textsuperscript{2+} channel different from that activated by TPA (21) in these cells. TPA at a concentration of 2 \mu M increased [Ca\textsuperscript{2+}], from 124 to 422 nM, while 2 \mu M TPA added to cells previously stimulated with forskolin for 15 min increased [Ca\textsuperscript{2+}], from 191 to 382 nM. A shorter preincubation with forskolin for 5 min prior to TPA stimulation resulted in an intermediate lag phase, rate of rise of [Ca\textsuperscript{2+}], and peak [Ca\textsuperscript{2+}], after 2 \mu M TPA when these characteristics of the rise in [Ca\textsuperscript{2+}] are compared to the absence of forskolin or forskolin preincubation for 15 min. The effect of PTH, which increased both [Ca\textsuperscript{2+}], and cAMP and probably also activates the protein kinase C (22, 23) in UMR-106 cells, on the TPA-mediated effect is shown in Fig. 9 (bottom panel). Preincubation of the cells with 2 units/ml bovine PTH (1-34) inhibited the TPA-mediated increase in [Ca\textsuperscript{2+}], in a time-dependent manner. Although forskolin increases cellular cAMP faster and to higher levels compared to PTH, the inhibition of the TPA-activated plasma membrane Ca\textsuperscript{2+} channel by PTH was more complete and faster compared to the forskolin effect on the same channel.

**DISCUSSION**

The tumor-promoting phorbol esters have been shown to substitute for the naturally occurring diacylglycerol in stimulating protein kinase C (2). They have been widely used to study the protein kinase C-mediated biological responses in a
variety of cells (1). Phorbol ester-dependent activation of protein kinase C was shown to inhibit hormone-regulated plasma membrane Ca\(^{2+}\) channels (5-7) and voltage-sensitive Cl\(^{-}\) (27) and Ca\(^{2+}\) currents in excitable tissues (8), to facilitate depolarization-activated Ca\(^{2+}\) currents in adrenal medulla, and finally, to activate Ca\(^{2+}\) currents in elasmobranch neurons (9-11). Although it is implied that activation of Ca\(^{2+}\) currents in these other systems resulted in a change in [Ca\(^{2+}\)], [Ca\(^{2+}\)], was not directly assessed. The results presented here demonstrate that phorbol esters directly activate a plasma membrane Ca\(^{2+}\) channel in a mammalian cell line, resulting in an increase in [Ca\(^{2+}\)]. The diverse and sometimes opposite effects of protein kinase C activation on channel activity and the biological response are probably the result of the different forms of protein kinase C in different cell types or even in the same cells (28, 29).

The TPA-dependent increase in [Ca\(^{2+}\)], required the presence of medium Ca\(^{2+}\) and could be inhibited by Ca\(^{2+}\) channel blockers. This channel showed some selectivity toward the Ca\(^{2+}\) channel blockers with nicardipine being the most potent blocker. In contrast relatively high concentrations of diltiazem had no substantial effect on blocking the TPA-activated Ca\(^{2+}\) channel. The effect of the phorbol esters was restricted to the biologically active phorbol esters, TPA and PDA, and to the diacylglycerol analogue, OAG. 4\(\alpha\)-Phorbol, which has not been shown to activate protein kinase C (2), did not activate the Ca\(^{2+}\) channel in these cells. Therefore, it is possible to conclude that activation of the protein kinase C was required for the activation of the Ca\(^{2+}\) channel.

The absolute dependence of the TPA-mediated increase in [Ca\(^{2+}\)], on the presence of medium Ca\(^{2+}\) and its complete inhibition by nicardipine was taken as evidence that the increase in [Ca\(^{2+}\)], is solely due to Ca\(^{2+}\) entry from medium into the cytosol. Also, in other cells, these criteria have been used to distinguish between the contribution of intracellular and extracellular pools of Ca\(^{2+}\) to the observed increase in [Ca\(^{2+}\)] (30-33). However, two pieces of evidence, one direct and one indirect, show that at least in UMR-106 cells the elevation of [Ca\(^{2+}\)], by Ca\(^{2+}\) entry from medium to cytosol may trigger Ca\(^{2+}\) release from intracellular stores. We have noticed that when increasing concentrations of TPA were used, the lag period before the rise in [Ca\(^{2+}\)], decreased, while the rate of Ca\(^{2+}\) entry and the extent of the elevation in [Ca\(^{2+}\)], increased. These observations do not reflect slower binding of TPA to its receptors since reducing medium Ca\(^{2+}\) concentration or treating the cells with submaximal concentrations of Ca\(^{2+}\) channel blockers resulted in an increased lag period, a slower rate of Ca\(^{2+}\) entry, and a smaller peak of [Ca\(^{2+}\)], even in the presence of maximal concentrations of TPA. In addition, we were able to titrate the amount of Ca\(^{2+}\) in the intracellular stores with the Ca\(^{2+}\) ionophore, ionomycin. Exposure of the cells to TPA resulted in a partial depletion of the intracellular Ca\(^{2+}\) stores, only when the medium contained CaCl\(_2\) during TPA stimulation. These two phenomena may be explained by Ca\(^{2+}\)-dependent Ca\(^{2+}\) release from intracellular stores. When higher concentrations of TPA or medium Ca\(^{2+}\) are used, more Ca\(^{2+}\) enters into the cytosol to trigger Ca\(^{2+}\) release from intracellular stores, thereby producing an apparent shorter lag period and thus facilitating the rate of increase in [Ca\(^{2+}\)], and finally allowing [Ca\(^{2+}\)], to rise to higher levels. A Ca\(^{2+}\)-dependent Ca\(^{2+}\) release mechanism has been described and characterized in the sarcoplasmic reticulum of skeletal muscle (34, 35), an organelle equivalent to the endoplasmic reticulum in nonexcitable cells. This potential finding of Ca\(^{2+}\)-mediated Ca\(^{2+}\) release from endo-plasmic reticulum in UMR-106 cells requires further investigation.

After the peak increase in [Ca\(^{2+}\)], following TPA stimulation, the partial reduction of [Ca\(^{2+}\)], is due to time-dependent inactivation of the TPA-stimulated Ca\(^{2+}\) channel. This is concluded from the failure of CaCl\(_2\) added to medium of prestimulated cells to induce the expected increase in [Ca\(^{2+}\)]. In addition, after prolonged stimulation with TPA, the cells were not able to reduce [Ca\(^{2+}\)], after addition of nicardipine or EGTA to the medium. The latter experiment also provides evidence that the partial reduction in [Ca\(^{2+}\)], was not due to a dye leak from the cells. EGTA added 1.5 min after TPA was able to reduce [Ca\(^{2+}\)], from 447 to 86 nM, while the same addition of EGTA 10 min after TPA stimulation only reduced [Ca\(^{2+}\)], from 297 to 211 nM. The concentration of EGTA used reduced medium Ca\(^{2+}\) to about 45 nM and, therefore, should quench most of the fluorescence of any extracellular dye that had leaked from the cells and allow the dye-containing cells to reduce [Ca\(^{2+}\)], back to 66 nM. In addition, measurements of medium dye content after 10 min of incubation at 37 °C with or without TPA show that dye leak was similar in both cells (about 2.5%). At present, it is not clear why [Ca\(^{2+}\)], was not reduced back to resting levels, despite the inactivation of the Ca\(^{2+}\) channel. However, it is unlikely that a second organic Ca\(^{2+}\) channel blocker-insensitive Ca\(^{2+}\) entry pathway was activated, since even after removal of medium Ca\(^{2+}\) the cells were not able to reduce [Ca\(^{2+}\)], back to the prestimulated level.

The inactivation of the channel also appears to be mediated by protein kinase C. Two possible mechanisms can lead to channel inactivation. One is down-regulation of the TPA receptors, and the other is direct inactivation of the channel. Down-regulation of the TPA receptors has been reported in several cell types (36-38). However, down-regulation required maximal occupancy of the receptors and long (about 1 h) incubation at 37 °C (36). In UMR-106 cells, incubation with 1 nM TPA for 10 min was sufficient to block the effect of 2 μM TPA. Further, elevation of cellular cAMP concentration also inhibited TPA effect on [Ca\(^{2+}\)]. Therefore, it appears that inactivation was not due to down-regulation of TPA receptors. It is more likely that protein kinase C or cAMP-dependent protein kinase was able to phosphorylate the channel and prevent its activation by TPA. Similar feedback inhibition by protein kinase C on protein kinase C-mediated responses has been described in a variety of cell types (5-7, 39-41). As reported here for UMR-106 cells, protein kinase C-mediated inhibition of protein kinase C-dependent responses was rapid and required submaximal concentrations of agonists like TPA.

Stimulation of UMR-106 cells with PTH is followed by a rapid transient increase in [Ca\(^{2+}\)], (Fig. 9), which we previously termed phases 1 and 2 of the PTH signal (21). This PTH-mediated increase in [Ca\(^{2+}\)], required medium Ca\(^{2+}\) and was blocked by nicardipine and verapamil, but not diltiazem. The rise in [Ca\(^{2+}\)], was blocked in a time- and concentration-dependent manner by preincubation of the cells with forskolin, dibutyryl cAMP, or low concentrations of PTH or TPA. The rapid transient PTH-activated channel was also rapidly inactivated (phase 2). All of these properties are identical to those described here for the TPA-mediated increase in [Ca\(^{2+}\)]. The only difference between the two agonists is that the maximal dose of TPA was more effective than the maximal dose of PTH in increasing [Ca\(^{2+}\)]. The close similarity between the [Ca\(^{2+}\)] signal induced by PTH and TPA and the ability of PTH to hydrolyze phosphatidylinositol phosphates (22, 23) suggest that phases 1 and 2 of the PTH-generated Ca\(^{2+}\) signal are mediated by activation of protein kinase C.
The functional significance of the protein kinase C-stimulated Ca\textsuperscript{2+} channel in these cells, in particular, and in osteoblasts in bone, in general, is presently unknown. It can be hypothesized that a protein kinase C-activated Ca\textsuperscript{2+} channel may be important in signal transduction rather than transcellular Ca\textsuperscript{2+} transport, since this channel is only short lived. The subsequent activation of the CAMP-dependent Ca\textsuperscript{2+} channel, which is a long-lived channel, may be important in signal transduction rather than transcellular transport.

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