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Phorbol Ester-Stimulated Bidirectional Transmembrane Calcium Flux in A_7r_5 Vascular Smooth Muscle Cells

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SUMMARY

In A_7r_5 vascular smooth muscle cells, the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) caused a marked increase in the rate of unidirectional Ca^{2+} influx and a 63 ± 9% increase in net 30-min $^{45}Ca^{2+}$ uptake. Maximal TPA-stimulated $^{45}Ca^{2+}$ uptake occurred at concentrations ≤ 3 nM and was equivalent to the maximal uptake stimulated by 55 mM KCl or 1 μM Bay k 8644. TPA-stimulated Ca^{2+} uptake was not additive to KCl- or Bay k 8644-stimulated uptake, and was inhibited by verapamil (100 μM), nitrendipine (1 μM), or high concentrations of Bay k 8644 (≥10 μM). The biologically inactive phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate methyl ether (10 nM) had no effect on $^{45}Ca^{2+}$ uptake. TPA caused a 43 ± 12% increase in Ca^{2+} efflux in 30 min, and exposure to TPA (10 nM) for 5 and 30 min caused no significant change in net cellular Ca^{2+} content as determined by radioisotopic equilibration or atomic absorption spectroscopy. TPA caused no apparent change in intracellular free Ca^{2+} concentration as determined with quin 2. Thus, in A_7r_5 cells, TPA causes a marked increase in Ca^{2+} influx through channels with the pharmacological characteristics of dihydropyridine-sensitive, voltage-dependent channels. This TPA-stimulated Ca^{2+} uptake is associated with increased Ca^{2+} efflux and no significant change in net cellular Ca^{2+} content or intracellular free Ca^{2+} concentration.

Stimulation of several Ca^{2+}-mobilizing receptors results in an increase in phosphoinositide turnover and the generation of two putative second messengers, inositol trisphosphate and DAG (1–5). Considerable evidence indicates that inositol trisphosphate causes the mobilization of intracellular Ca^{2+} stores and is a likely mediator of the initial rapid rise in intracellular Ca^{2+} concentration. DAG is a neutral lipid that, in the presence of Ca^{2+}, greatly increases the activity of Ca^{2+}/phospholipid-dependent PK-C (2–5). PK-C can also be stimulated directly by the tumor-promoting phorbol esters (2–6). It has been proposed that DAG, through activation of PK-C, may mediate the sustained phase of the cellular response in a variety of tissues, including vascular smooth muscle (7, 8).

Phorbol esters cause a slowly developing contraction of vascular smooth muscle, presumably due to activation of PK-C (9–11). This phorbol ester-stimulated contraction is inhibited by Ca^{2+} channel antagonists and facilitated by the Ca^{2+} channel agonist Bay k 8644 (11). Likewise, PK-C activation by phorbol ester, or the direct intracellular injection of activated PK-C, results in an increase in inward Ca^{2+} conductance in neurons (12) and photoreceptor cells (13), respectively. Based on these observations, we performed experiments in A_7r_5 cells, a clonal cell line originally derived from fetal rat thoracic aorta vascular smooth muscle (14–16), to determine whether phorbol esters cause an increase in Ca^{2+} influx; and, if so, (a) to characterize the nature of the influx channel, and (b) to determine the effect of phorbol ester-stimulated Ca^{2+} influx on net cellular Ca^{2+} balance. In A_7r_5 cells, we evaluated the effects of TPA on Ca^{2+} influx and efflux, net cellular Ca^{2+} content, and intracellular free Ca^{2+} concentration.

TPA increased both the rate of unidirectional Ca^{2+} influx and the net $^{45}Ca^{2+}$ uptake at 30 min (maximal effect, ≤ 3 nM). The increase in net $^{45}Ca^{2+}$ uptake at 30 min was equivalent to that caused by 55 mM KCl or the Ca^{2+} channel agonist Bay k 8644 (1 μM), and was fully inhibited by verapamil (100 μM), nitrendipine (1 μM), or high concentrations by Bay k 8644 (≥10 μM). TPA-stimulated $^{45}Ca^{2+}$ influx was associated with an increase in $^{45}Ca^{2+}$ efflux, but no change in net cellular Ca^{2+} content determined by $^{45}Ca^{2+}$ isotopic equilibrium or atomic absorption spectroscopy. TPA (10 nM) had no effect on intracellular free Ca^{2+} concentration determined with quin 2. Thus, these data show that, in A_7r_5 cells, the phorbol ester TPA causes Ca^{2+} influx through channels with the pharmacologic characteristics of dihydropyridine-sensitive, voltage-dependent Ca^{2+} influx channels, and suggest that phorbol esters induce a

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ABBREVIATIONS: DAG, diacylglycerol; PK-C, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; quin 2, 2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline; BSS, balanced salt solution; HEPES, 4-(2-hydroxyethyl ether)-1-piperazineethanesulfonic acid.

sustained bidirectional transmembrane Ca^{2+} flux that is associated with little or no net change in total cellular Ca^{2+} content.

Materials and Methods

Cell culture. The A_7r_5 rat aortic smooth muscle cell line, as originally established and characterized by Kimes and Brandt (14), was obtained from the American Type Culture Collection, Bethesda, MD. Cells were plated in plastic culture dishes (Nunc), at an initial density of 5000–9000 cells/cm^2, and grown in Dulbecco's modified Eagle's medium (Gibco) containing antibiotics and 10% fetal calf serum (Gibco) in a humidified atmosphere (37°) under 5% CO_2/95% air. Cells reached confluence at approximately 4 days and were used for studies between 5 and 8 days, at which time there were approximately 35,000 cells/cm^2. Cells used for experiments were from passages 6 through 30, during which time they exhibited stable morphologic characteristics by light microscopy and a consistent degree of $^{45}Ca^{2+}$ uptake in response to 55 mM KCl.

Ca^{2+} uptake. $^{45}Ca^{2+}$ uptake was measured in BSS consisting of: 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 5 mM HEPES at pH 7.40. The depolarizing solution differed only in that NaCl was 95 mM and KCl was 55 mM. Confluent cells on 35-mm plastic dishes were washed three times with BSS at 37°, and the uptake period was initiated by addition of 1 ml of BSS or depolarizing solution containing approximately 1.5 μCi of $^{45}Ca^{2+}$ and the agent(s) under study. Uptake was terminated at various time intervals by rapidly washing each plate five times with 2 ml of ice-cold modified BSS which contained 10 mM $LaCl_3$ and no $CaCl_2$. Cells were allowed to stand for 10 min at 4° in the last wash aliquot, after which the solution was aspirated and the cells were dried at room temperature for 20 min. Cells were then dissolved in 0.1 N HNO_3 (20 min, 4°) and transferred to scintillation vials to which 13 ml of scintillation cocktail were added (Liquiscint, National Diagnostics).

Ca^{2+} efflux. Confluent cells on 35-mm plastic dishes were equilibrated for 18–24 hr with 2 ml of fresh culture medium containing 2 μCi/ml of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ efflux was initiated by washing the cells three times with BSS at 37°. The assay was terminated at various time intervals by washing the cells four times with ice-cold modified BSS containing 10 mM $LaCl_3$ and no $CaCl_2$, followed by an additional 10-min incubation in 2 ml of the same solution. Cell-associated $^{45}Ca^{2+}$ was then determined as described for the Ca^{2+} uptake assay above.

Cellular Ca^{2+} content at isotopic equilibrium. Confluent cells on 35-mm dishes were equilibrated for 18–24 hr with 2 ml of fresh culture medium containing 2 μCi/ml $^{45}Ca^{2+}$. The assay was initiated by applying 200 μl of fresh culture medium containing $^{45}Ca^{2+}$ (2 μCi/ml) plus appropriate agents. At various time intervals, the assay was terminated by washing each dish five times with BSS containing 10 mM $LaCl_3$ and no $CaCl_2$; cell-associated $^{45}Ca^{2+}$ was determined as described above.

Quin 2. Intracellular free Ca^{2+} concentration was measured with quin 2 using methods similar to those described by Ambler and Taylor (17). Confluent cells attached to 13.5 × 27 mm glass coverslips were loaded with quin 2 by placing the coverslips in 35-mm dishes containing 2 ml of BSS plus 50 μM quin 2/AM for 30 min at 37°. Free quin 2 was removed by washing the cells twice with 2 ml of culture medium containing 10% fetal calf serum at 37°. Immediately prior to starting the assay, coverslips were washed once with BSS at 37° and placed in a specially adapted 3-ml quartz curvette (Fischer Scientific) equipped with a stirring apparatus. The coverslip was oriented so that cells faced the excitation beam. Quin 2 fluorescence was determined in a SPEX model CM-1 Fluorolog-2 spectrofluorimeter with thermostatic control, and fluorescence (excitation, 339 nm; emission, 492 nm) was calibrated using $MnCl_2$ (0.5 mM) and digitonin (10 μg/ml). Cells remained attached to the coverslip during digitonin exposure, as assessed by microscopic examination before and after the experiment, and the fluorescence signal caused by digitonin was stable over several minutes.

Cellular Ca^{2+} content by atomic absorption spectroscopy. Confluent cells on 35-mm dishes were washed three times with BSS at 37°. BSS or depolarizing solution was then added and the dishes were incubated at 37° in a water bath. At various time intervals, the assay was terminated by rapidly washing each dish five times with 2 ml of ice-cold modified BSS containing 10 mM $LaCl_3$ and no $CaCl_2$. Cells from three plates were dissolved in 0.1 N HNO_3 (20 min, 4°) and pooled in a total volume of 0.6 ml. Atomic absorption spectroscopy was performed on a 0.5-ml aliquot in a Perkin-Elmer model 5000 spectrometer with absorbance at 212.8 nm, and compared to a Ca^{2+} standard dilution curve freshly prepared to 0.1 N HNO_3. Cellular Ca^{2+} content was expressed as nmol of Ca^{2+}/cm^2 of culture dish surface area.

Drugs. $^{45}Ca^{2+}$ was obtained from ICN, Irvine, CA. Quin 2/AM was obtained from Calbiochem, San Diego, CA. Nitrendipine, Bay k 8644, and verapamil were generously supplied by Miles Pharamceuticals (West Haven, CT), Bayer AG (Wuppental, FRG), and Searle Pharmaceuticals (Chicago, IL), respectively. TPA, TPA-methyl ether, dimethyl sulfoxide, and digitonin were obtained from Sigma. TPA and TPA-methyl ether were dissolved in dimethyl sulfoxide to a final concentration of 1:10,000 at a TPA or TPA-methyl ether final concentration of 10 nM. Accordingly, in all experiments involving phorbol esters, control cells were incubated with dimethyl sulfoxide at a final concentration of 1:10,000.

Results

Effects of KCl and TPA on $^{45}Ca^{2+}$ uptake. Compared to basal conditions, KCl (55 mM) and TPA (10 nM) each caused a marked increase in net $^{45}Ca^{2+}$ uptake (Fig. 1A). At 30 min, KCl (55 mM) and TPA (10 nM) increased net $^{45}Ca^{2+}$ uptake by 64 ± 8% ($p < 0.01$ versus basal; $n = 3$) and 63 ± 9% ($p < 0.01$ versus basal; $n = 12$), respectively. At the end of 24 hr, the $^{45}Ca^{2+}$ content of TPA-stimulated (10 nM) cells was 101 ± 4% of that in control cells (p = not significant versus control; $n = 4$).

The TPA-stimulated increase in net 30-min $^{45}Ca^{2+}$ uptake was concentration related, with maximal uptake occurring at ≤ 3 nM (Fig. 2A). The effect of TPA on net 30-min $^{45}Ca^{2+}$ uptake was inhibited by both nitrendipine and verapamil, with complete inhibition at concentrations of 1 μM and 100 μM, respectively (Fig. 2B). KCl-stimulated net 30-min $^{45}Ca^{2+}$ uptake was fully inhibited by 1 μM nitrendipine with an IC_{50} of 3 nM ($n = 3$), in good agreement with the reported IC_{50} for inhibition of KCl-stimulated $^{45}Ca^{2+}$ influx and contraction in rabbit aortic rings (18).

The maximal increases in net 30-min $^{45}Ca^{2+}$ uptake caused by 55 mM KCl and 10 nM TPA were similar, and net $^{45}Ca^{2+}$ uptake caused by KCl and TPA together was no greater than that caused by either alone (Fig. 3). Likewise, net 30-min $^{45}Ca^{2+}$ uptake caused by KCl and TPA, alone and together, was totally inhibited by nitrendipine (1 μM) or verapamil (100 μM) (Fig. 3). The biologically inactive phorbol ester, TPA-methyl ether (10 nM), had no effect on $^{45}Ca^{2+}$ uptake (Fig. 3). Nitrendipine (1 μM) caused a small decrease in basal $^{45}Ca^{2+}$ uptake (*CON+NIT*, Fig. 3), suggesting that under the basal conditions of this study there is a small degree of dihydropyridine-sensitive $^{45}Ca^{2+}$ uptake.

To evaluate unidirectional Ca^{2+} influx, the initial 5-min $^{45}Ca^{2+}$ uptake rate was determined following a 30-min preincubation with TPA (Fig. 1B). Over this time period, TPA-stimulated $^{45}Ca^{2+}$ uptake was linear ($r = 0.98$). Basal $^{45}Ca^{2+}$ influx was 0.006 ± 0.037 nmol/10^6 cells/min and increased to 0.335 ± 0.010 nmol/10^6 cells/min ($p < 0.025$ versus basal; $n = 3$) following a 30-minute preincubation with 10 nM TPA. In the absence of preincubation with TPA, the increase in $^{45}Ca^{2+}$

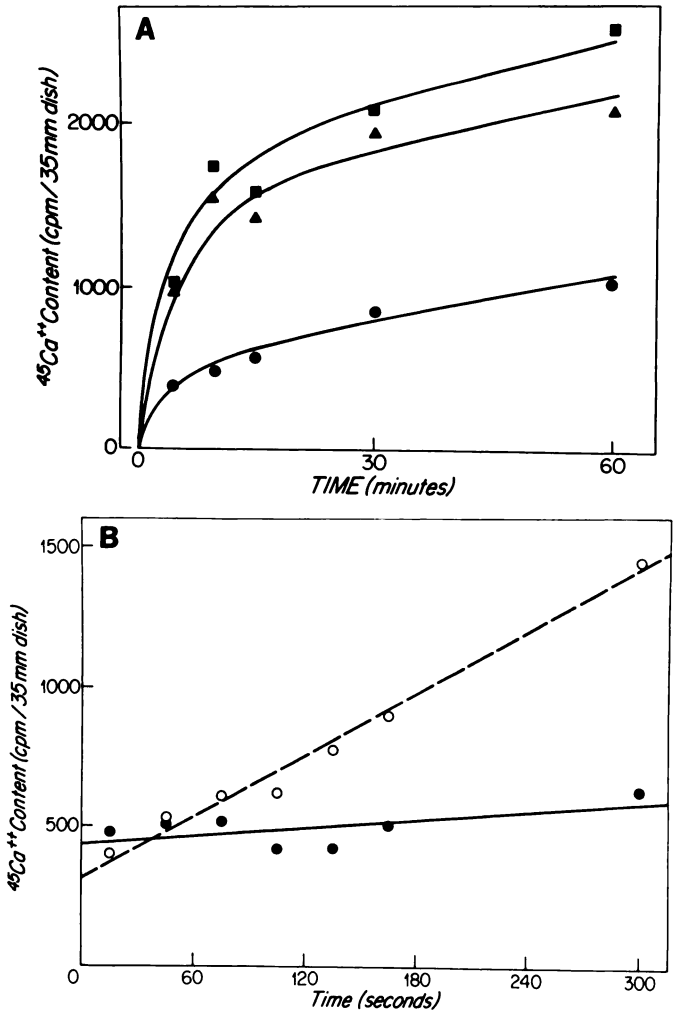

Fig. 1. Effects of TPA and KCl on Net $^{45}Ca^{2+}$ uptake (A) and the effect of TPA on unidirectional $^{45}Ca^{2+}$ uptake (B). A. Net $^{45}Ca^{2+}$ uptake was determined under basal conditions (●) and in response to 10 nM TPA (■) and 55 mM KCl (▲). The experiment shown is typical of three such experiments, each performed in triplicate. B. Unidirectional Ca^{2+} influx rate determined during the first 5 min of $^{45}Ca^{2+}$ uptake. In the experiment depicted, the basal (●) Ca^{2+} influx rate was 0.031 nmol/10^6 cells/min, and increased to 0.240 nmol/10^6 cells/min following a 30-min preincubation with 10 nM TPA (○). The experiment shown is typical of three such experiments, each performed in triplicate.

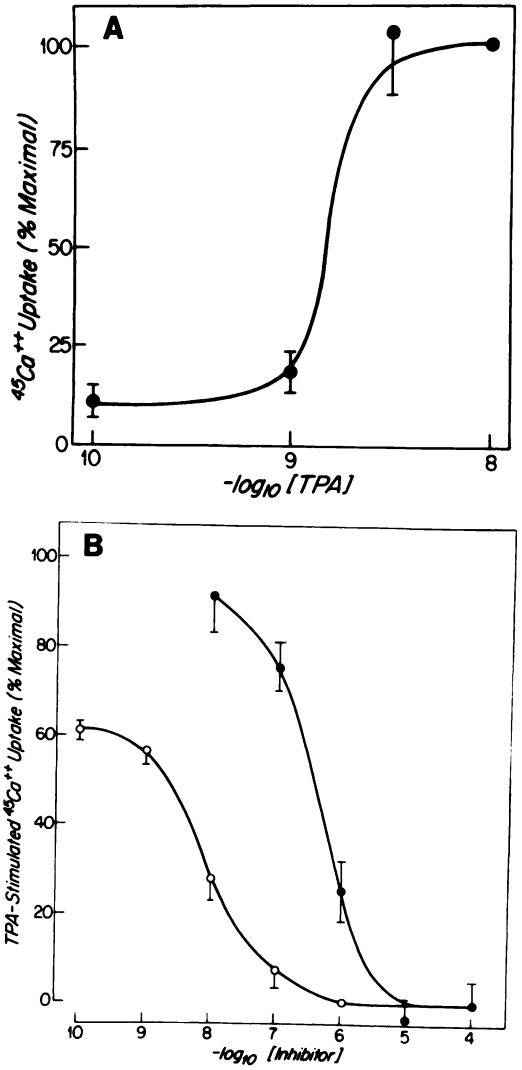

Fig. 2. Concentration dependence of the effect of TPA on net $^{45}Ca^{2+}$ uptake (A), and inhibition of maximal TPA-stimulated (10 nM) net 30-min $^{45}Ca^{2+}$ uptake by nitrendipine (○) or verapamil (●) (B). The data depicted are the mean ± standard error of three to six experiments, each performed in triplicate.

uptake rate lagged approximately 3 min after addition of TPA (data not shown).

Effect of Bay k 8644 on $^{45}Ca^{2+}$ uptake. The Ca^{2+} channel agonist Bay k 8644 caused a concentration-related increase in net $^{45}Ca^{2+}$ uptake, with the maximal effect occurring at 1 μM (Fig. 4). Maximal TPA (10 nM)- and Bay k 8644 (1 μM)-stimulated Ca^{2+} uptakes were similar in magnitude, and the two agents together caused no greater uptake than either alone. At Bay k 8644 concentrations of ≥10 μM, there was marked inhibition of maximal TPA-stimulated $^{45}Ca^{2+}$ uptake (−76 ± 2%, $p < 0.05$ versus TPA alone; $n = 4$) (Fig. 4).

Effect of TPA on $^{45}Ca^{2+}$ efflux, net cellular Ca^{2+} content, and $[Ca^{2+}]_i$. In cells equilibrated with $^{45}Ca^{2+}$ for 18–24 hr, TPA (10 nM) caused an approximately 34 ± 12% decrease ($p < 0.025$; $n = 3$) in $^{45}Ca^{2+}$ content at 30 min (Fig. 5). The magnitude of TPA-stimulated 30-min $^{45}Ca^{2+}$ efflux was similar to that caused by 55 mM KCl (≥29 ± 1%; $n = 3$) (Fig. 5, *inset*).

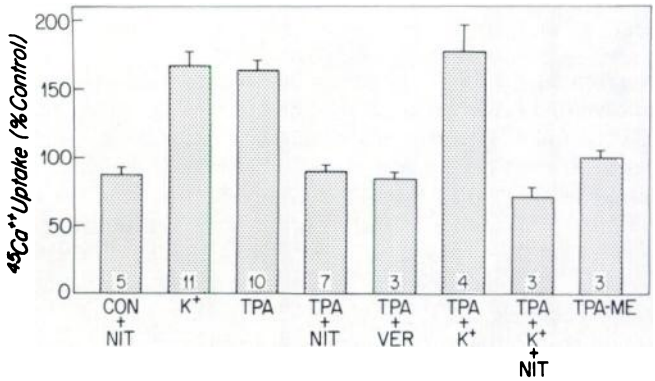

Fig. 3. Effects of various stimulators and inhibitors of Ca^{2+} flux on net 30-min $^{45}Ca^{2+}$ uptake. Data are expressed as percentage of basal $^{45}Ca^{2+}$ uptake, and represent the mean ± standard error of the indicated number of experiments, each performed in triplicate. *CON*, control; *NIT*, 1 μM nitrendipine; K^+, 55 mM KCl; TPA, 10 nM; *VER*, 100 μM verapamil; TPA-ME, 10 nM TPA methyl ether.

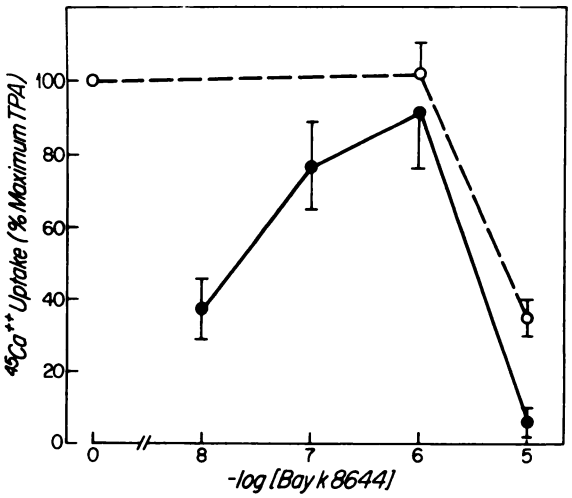

Fig. 4. Concentration dependence of the effects of Bay k 8644 on basal (●) and maximal (○) TPA-stimulated (10 nM) net 30-min $^{45}Ca^{2+}$ uptake. Data are presented as a percentage of maximal TPA-stimulated uptake and represent three to six experiments performed in triplicate.

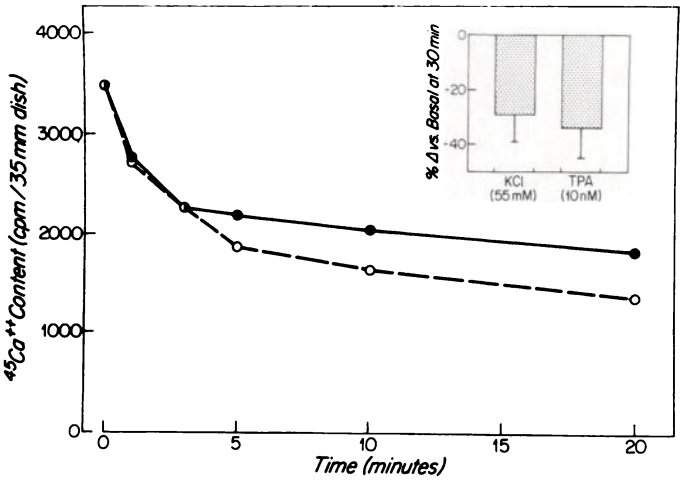

Fig. 5. Effect of TPA (10 nM) on $^{45}Ca^{2+}$ efflux. $^{45}Ca^{2+}$ efflux under basal conditions (●) and in response to 10 nM TPA (○). TPA-stimulated $^{45}Ca^{2+}$ efflux at 30 min was similar in magnitude to that caused by 55 mM KCl (n = 3, p = not significant; *inset*). The efflux time course depicted is typical of three such experiments, each performed in triplicate.

After the addition of TPA, there was an approximately 3-min delay before there was an increase in $^{45}Ca^{2+}$ efflux.

To determine changes in net cellular Ca^{2+} content, cells equilibrated for 18–24 hr in medium containing $^{45}Ca^{2+}$ were exposed to TPA in the continued presence of $^{45}Ca^{2+}$ at isotopic equilibrium. At isotopic equilibrium, total cellular Ca^{2+} content was 9.13 ± 1.12 nmol/mg (n = 5). There was no significant change in net $^{45}Ca^{2+}$ content following a 5- or 30-min exposure to 10 nM TPA, with net $^{45}Ca^{2+}$ content of TPA-stimulated cells equal to 102.3 ± 2.4 and 101.6 ± 7.3% of that in control cells at 5 and 30 min, respectively (n = 5; p = not significant for both). Likewise, total cellular Ca^{2+} content determined by atomic absorption spectroscopy was unchanged following exposure to TPA (10 nM) for 5 or 30 min (control) cells = 0.165 ± 0.15 nmol of Ca^{2+}/cm^2; TPA, 5 min = 0.161 ± 0.009 nmol of Ca^{2+}/cm^2; TPA, 30 min, 0.171 ± 0.051 nmol of Ca^{2+}/cm^2; n = 3, p = not significant). Basal $[Ca^{2+}]_i$ in cells loaded with quin 2 was 107 ± 17 nM (n = 5). TPA (10 nM) caused no significant increase in $[Ca^{2+}]_i$ during experiments monitored for up to 12 min (peak $[Ca^{2+}]_i$ after TPA = 141 ± 44 nM; p = not significant versus basal; n = 5), whereas ionomycin (20 μM) caused an increase in fluorescence similar to that caused by digitonin (n = 3).

Discussion

These data show that in A_7r_5 cells the phorbol ester TPA stimulates Ca^{2+} influx through channels that have the pharmacologic characteristics of voltage-dependent channels. This conclusion is based on the following observations. First, TPA-stimulated Ca^{2+} influx is fully inhibited by appropriate concentrations of two classes of inhibitors of voltage-dependent Ca^{2+} channels. Second, maximal KCl and TPA-stimulated Ca^{2+} influx are of similar magnitude, are not additive, and, together, are fully blocked by nitrendipine. Third, maximal TPA-stimulated Ca^{2+} influx is similar to that caused by the Ca^{2+} channel agonist Bay k 8644, the effects of TPA and Bay k 8644 together are not additive, and at high concentrations Bay k 8644 causes inhibition of TPA-stimulated Ca^{2+} influx. These pharmacologic observations strongly suggest that TPA-induced calcium influx is via dihydropyridine-sensitive, voltage-dependent Ca^{2+} channels. Both rapidly inactivating, dihydropyridine-insensitive (T-type) and slowly inactivating, dihydropyridine-sensitive (L-type) Ca^{2+} channels have been identified in rat mesenteric artery vascular smooth muscle cells (19) and A-10 cells (20), the latter closely related to the A_7r_5 cells described in this report (14). Although the effects of nitrendipine and Bay k 8644 suggest that TPA-stimulated Ca^{2+} influx is primarily by way of L-type channels (21), precise characterization of the channel type will require electrophysiologic study.

Rasmussen *et al.* (9), Danthuluri and Deth (10), and others (22–25) have observed that phorbol esters cause a lowly developing contraction in vascular smooth muscle. Most (9–11, 22), but not all (23) studies have suggested that TPA-induced vascular contraction is dependent on external Ca^{2+}, since it is abolished or attenuated by removal of extracellular Ca^{2+}. An important role for extracellular Ca^{2+} is further suggested by the finding that the time to onset and amplitude of TPA-induced contraction is markedly influenced by agents that alter transmembrane Ca^{2+} flux: increased extracellular Ca^{2+} and the Ca^{2+} channel agonist Bay k 8644 cause a shortening of the time to onset of TPA-induced contraction, whereas the Ca^{2+} channel inhibitor nitrendipine causes a substantial reduction in the amplitude of TPA-induced contraction (11). Thus, the observation in this study that TPA causes an increase in Ca^{2+} influx through voltage-dependent channels is consistent with prior studies that indicated a strong relationship between TPA-induced contraction and Ca^{2+} influx and supports the suggestion that Ca^{2+} influx may play a role in mediating TPA-induced contraction.

The mechanism by which TPA causes increased Ca^{2+} influx remains to be determined. It is highly likely that the effects of TPA in this study are due to activation of PK-C, since these effects occurred at low concentrations consistent with the affinity of this phorbol ester for PK-C (6) and were not caused by a biologically inactive phorbol ester. PK-C activation is well known to cause phosphorylation of several membrane components (26–29) and, therefore, may act directly or indirectly on the voltage-dependent channel to cause an increase in Ca^{2+} conductance independent of a change in membrane potential. Consistent with this suggestion, recent patch-clamp studies indicate that TPA or intracellular injection of activated PK-C

causes an increase in inward Ca^{2+} conductance in neurons (12) and photoreceptor cells (13). However, in embryonic chicken dorsal root ganglion neurons, phorbol esters cause a decrease in inward Ca^{2+} conductance (30), indicating that there may be important species- and/or tissue-specific differences in the actions of phorbol esters on Ca^{2+} flux. Alternatively, phorbol esters might alter Ca^{2+} flux due to an effect on Na^+/H^+ exchange, secondarily affecting Na^+/Ca^{2+} exchange. However, this seems unlikely since phorbol ester-induced Ca^{2+} influx in A_7r_5 cells appears to be through voltage-dependent channels which are not known to be involved in the process of Na^+/Ca^{2+} exchange. It is also possible that phorbol esters stimulate Ca^{2+} influx by causing cellular depolarization. In support of this possibility, phorbol esters have been shown to inhibit a voltage-dependent K^+ current, the M-current, in a neuronal cell (31). It cannot be assumed that these observations in A_7r_5 cells accurately reflect events in vascular smooth muscle. Nevertheless, this convenient cell line should prove useful for further assessment of the ionic events associated with activation of PK-C.

Despite causing a substantial increase in unidirectional Ca^{2+} influx, there is no apparent change in net cellular Ca^{2+} content at 5 or 30 min as detected by the radioisotopic equilibrium method or atomic absorption spectroscopy. This suggests that, at the relatively late times evaluated in this study, phorbol ester-stimulated Ca^{2+} influx and efflux are balanced. Consistent with this suggestion, TPA caused a substantial increase in $^{45}Ca^{2+}$ efflux and, likewise, TPA-stimulated Ca^{2+} influx was not associated with a significant increase in free intracellular Ca^{2+} concentration as detected by quin 2. Other possible reasons for the failure to observe an increase in quin 2 fluorescence include Ca^{2+} buffering by quin 2 and the localized sequestration of Ca^{2+} in pools not freely accessible to the cytoplasm. However, the quin 2 data, taken together with the lack of an increase in net cellular Ca^{2+} content and evidence of increased Ca^{2+} efflux, suggest that TPA-stimulated Ca^{2+} influx is largely balanced by efflux. The mechanism responsible for TPA-induced Ca^{2+} efflux cannot be determined from these data. However, the finding of increased Ca^{2+} efflux in the absence of an increase in intracellular free Ca^{2+} concentration raises the possibility that TPA acts directly to stimulate Ca^{2+}/Mg^{2+} ATPase, a mechanism previously observed in rabbit neutrophils (32).

There is substantial evidence that the inositol trisphosphate and DAG arms of hormone-stimulated phosphoinositide turnover may act synergistically (2–5). In addition, Rasmussen and colleagues have further hypothesized, based in part on studies of the angiotensin II-stimulated secretion of aldosterone by adrenal glomerulosa cells, that DAG is responsible for the sustained phase of the cellular response (7, 8, 33). According to this thesis, the initial hormone-stimulated increases in intracellular Ca^{2+} concentration and DAG promote the conversion of inactive, Ca^{2+}-insensitive PK-C to an active plasma membrane-associated, Ca^{2+}-sensitive form of the enzyme, the activity of which is regulated by the rate of Ca^{2+} influx across the plasma membrane. In adrenal glomerulosa cells, it was also observed that, although Ca^{2+} influx is essential for maintenance of tonic angiotensin II-stimulated aldosterone secretion, there was no increase in total cellular Ca^{2+} content, leading to the further suggestion that the increased Ca^{2+} influx during tonic aldosterone secretion was associated with increased bidirectional transmembrane Ca^{2+} cycling, most likely from a submembrane domain. Our data provide support for this general thesis and further suggest that activation of PK-C can cause a marked increase in bidirectional Ca^{2+} flux. One implication of these findings is that PK-C, once activated, might then act to perpetuate its sustained activation by causing an increase in bidirectional transmembrane Ca^{2+} flux. According to this model, the sustained activation of PK-C would depend importantly on the continued hormone-stimulated production of DAG (33, 34).

References

1. Berridge, M. J., and R. F. Irvine. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature (Lond.)* **312**:315–321 (1984).
2. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.)* **308**:693–698 (1984).
3. Exton, J. H. Role of calcium and phosphoinositide in the actions of certain hormones and neurotransmitters. *J. Clin. Invest.* **75**:1753–1757 (1985).
4. Abdel-Latif, A. A. Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacol. Rev.* **38**:227–272 (1986).
5. Rasmussen, H. The calcium messenger system. *N. Engl. J. Med.* **314**:1094–1101, 1164–1170 (1986).
6. Castagna, M., Y. Takai, K. Taibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257**:1847–1851 (1982).
7. Rasmussen, H., and P. W. Barrett. Calcium messenger system: an integrated view. *Physiol. Rev.* **64**:938–984 (1984).
8. Kojima, I., K. Kojima, D. Kreutter, and H. Rasmussen. The temporal integration of the aldosterone secretory response to angiotensin occurs via two intracellular pathways. *J. Biol. Chem.* **259**:14448–14457 (1984).
9. Rasmussen, H., J. Forder, Kojima, and A. Scriabine. TPA-induced contraction of isolated rabbit vascular smooth muscle. *Biochem. Biophys. Res. Commun.* **122**:776–784 (1984).
10. Danthuluri, N. R., and R. C. Deth. Phorbol ester-induced contraction of arterial smooth muscle and inhibition of alpha-adrenergic response. *Biochem. Biophys. Res. Commun.* **125**:1103–1109 (1984).
11. Forder, J., A. Scriabine, and H. Rasmussen. Plasma membrane calcium flux, protein kinase C activation and smooth muscle contraction. *J. Pharmacol. Exp. Ther.* **235**: 267–273 (1985).
12. DeRiemer, S. A., J. A. Strong, K. A. Albert, D. Greengard, and L. K. Kaczmarek. Enhancement of calcium current in *Aplysia* neurones by phorbol ester and protein kinase C. *Nature (Lond.)* **313**:313–316 (1985).
13. Farley, J., and S. Auerbach. Protein kinase C activation induces conductance changes in *Hermissenda* photoreceptors like those seen in associative learning. *Nature (Lond.)* **319**:220–223 (1985).
14. Kimes, B. W., and B. L. Brandt. Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Exp. Cell Res.* **98**:349–366 (1976).
15. Ruegg, U. T., V. M. Doyle, J. F. Zuber, and R. P. Hof. A smooth muscle cell line suitable for the study of voltage sensitive calcium channels. *Biochem. Biophys. Res. Commun.* **130**:447–453 (1985).
16. Kongsamut, S., S. B. Freedman, and R. J. Miller. Dihydropyridine sensitive calcium channels in a smooth muscle cell line. *Biochem. Biophys. Res. Commun.* **127**:71–79 (1985).
17. Ambler, S. K., and P. Taylor. Mobilization on intracellular calcium by alpha-1 adrenergic receptor activation in muscle cell monolayers. *J. Biol. Chem.* **261**:5866–5871 (1986).
18. Scriabine, A., C. E. Johnson, C. L. Anderson, O. S. Steinsland, and R. A. Janis. Ca^{2+} channel inhibitory effects of nitrendipine on isolated rabbit arteries, in *Nitrendipine* (A. Scriabine, S. Vanor, and K. Deck, eds.). Urban and Schwarzenberg, Baltimore, 283–295 (1984).
19. Bean, B. P., M. Sturek, A. Puga, and A. Hermsmeyer. Calcium channels in muscle cells isolated from rat mesenteric arteries: modulation by dihydropyridines. *Circ. Res.* **59**:229–235 (1986).
20. Friedman, M. E., G. Suarez-Kurtz, G. J. Kaczorowski, G. M. Katz, and J. P. Reuben. Two calcium currents in a smooth muscle cell line. *Am. J. Physiol.* **250**:H699–H703 (1986).
21. Nowycky, M. C., A. P. Fox, and R. W. Tsien. Three types of neuronal calcium channels with different calcium agonist sensitivity. *Nature (Lond.)* **316**:440–443 (1985).
22. Gleason, M. M., and S. F. Flaim. Phorbol ester contracts rabbit thoracic aorta by increasing intracellular calcium and by activating calcium influx. *Biochem. Biophys. Res. Commun.* **138**:1362–1369 (1986).
23. Sybertz, E. J., D. M. Desiderio, G. Tetzloff, and P. J. S. Chiu. Phorbol dibutyrate contractions in rabbit aorta: calcium dependence and sensitivity to nitrovasodilators and 8-BR-cyclic GMP. *J. Pharmacol. Exp. Ther.* **239**:78–83 (1986).
24. Chatterjee, M., and M. Tejada. Phorbol ester-induced contraction in chemically-skinned vascular smooth muscle. *Am. J. Physiol.* **251**:C356–C361 (1986).
25. Miller, J. R., D. J. Hawkins, and J. N. Wells. Phorbol diesters alter the

contractile responses of porcine coronary artery. *J. Pharmacol. Exp. Ther.* **239**:38–42 (1982).
26. Jacobs, S., N. E. Sahyoun, R. Saltiel, and P. Cuatrecasas. Phorbol esters stimulate phosphorylation of receptors for insulin and somatomedin-C. *Proc. Natl. Acad. Sci. USA* **80**:6211–6213 (1983).
27. Cochet, C., G. M. N. Gill, J. Meisenhelder, J. A. Cooper, and T. Hunter. C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine kinase activity. *J. Biol. Chem.* **259**:2553–2558 (1984).
28. Sibley, D. R., P. Nambi, J. R. Peters, and R. J. Lefkowitz. Phorbol esters promote beta-adrenergic receptor phosphorylation and adenylate cyclase desensitization in duck erythrocytes. *Biochem. Biophys. Res. Commun.* **121**:973–979 (1984).
29. Leeb-Lundberg, L. M., S. Cotecchia, J. W. Lomasney, J. F. DeBernardis, R. J. Lefkowitz, and M. G. Carom. Phorbol esters promote alpha-1 adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. USA* **82**:5651–5655 (1985).
30. Rane, S. G., and K. Dunlap. Kinase C activator 1,2-oleoylacetyl-glycerol attenuates voltage-dependent calcium current in sensory neurons. *Proc. Natl. Acad. Sci. USA* **83**:184–188 (1986).
31. Higashida, H., and D. A. Brown. Two polysphatidylinositide metabolites control two K^+ currents in a neuronal cell. *Nature (Lond.)* **323**:333–335 (1986).
32. Lagast, H., T. Pozzan, F. A. Waldvogel, and D. P. Lew. Phorbol myristate acetate stimulates ATP-dependent calcium transport by plasma membrane of neutrophils. *J. Clin. Invest.* **73**:878–883 (1984).
33. Kojima, I., K. Kojima, and H. Rasmussen. Role of calcium fluxes in the sustained phase of angiotensin II-mediated aldosterone secretion from adrenal glomerulosa cells. *J. Biol. Chem.* **260**:9177–9184 (1985).
34. Griendling, K. K., S. E. Rittenhouse, T. A. Brock, C. W. Powers, M. A. Gimbrone, Jr., and R. W. Alexander. Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J. Biol. Chem.* **261**:5901–5906 (1986).

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