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Protein kinase-C mediates dual modulation of L-type Ca^{2+} channels in human vascular smooth muscle

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Abstract

The role of protein kinase C (PKC) in cellular regulation of L-type Ca^{2+} channels was investigated in human umbilical vein smooth muscle. Activation of PKC, by low concentrations (< 30 nM) of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) caused inhibition of Ca^{2+} channels, while higher concentrations of TPA (>100 nM) elicited a transient rise, followed by sustained inhibition of Ca^{2+} channel activity in cell-attached patches. Low TPA concentrations predominantly reduced channel availability, while high concentrations of TPA (100 nM) transiently increased channel availability and, in addition, prolonged mean open time. The inactive 4-α-phorbol-12,13-didecanoate failed to affect channel activity, and pretreatment of the cells with PKC inhibitors (H-7, chelerythrine) antagonized inhibitory and stimulatory effects of TPA. Our results provide evidence for two distinct PKC-dependent mechanisms of L-type Ca^{2+} channel regulation in smooth muscle.

Key words: L-type calcium channel; Cellular regulation; Phorbol ester; Protein kinase C

1. Introduction

Calcium- and phospholipid-dependent protein kinase (PKC) has been suggested to play a critical role in smooth muscle Ca^{2+} homeostasis and regulation of contractility [1,2]. Among the various membrane transport systems which serve Ca^{2+} homeostasis, voltage-dependent Ca^{2+} channels have been implicated as one likely target of PKC regulation. Measurements of $^{45}Ca^{2+}$ influx and membrane currents in a variety of tissues, including also smooth muscle, have provided substantial evidence for PKC-dependent modulation of L-type channels [3–8]. However, the relationship between cellular PKC activity and voltage-dependent Ca^{2+} entry in smooth muscle is still a matter of controversy. The use of tumor promoting phorbol ester as experimental tool to activate PKC in smooth muscle has yielded divergent results, providing evidence for both phorbolester-induced facilitation [3,5] as well as inhibition of voltage-dependent Ca^{2+} entry [4,9]. Although contrasting in nature, the available evidence unequivocally demonstrate PKC-dependent regulation of smooth muscle L-type Ca^{2+} channels as a central albeit complex mechanism which links signal input from a variety of hormone receptors to Ca^{2+} entry. Thus, PKC may be of particular importance for fine adjustment of Ca^{2+} channel activity by hormones and neurotransmitter. Until now, PKC-dependent modulation of single smooth muscle Ca^{2+} channels in intact cells has not been investigated. In this study we have analyzed changes in L-type Ca^{2+} channel function, induced by phorbol ester in intact smooth muscle cells isolated from human umbilical vein, and present evidence for a dual PKC-dependent channel regulation.

2. Materials and methods

2.1. Cell preparation

Single smooth muscle cells were enzymatically isolated from the media of human umbilical veins. Vessels were cannulated, and perfused with HEPES buffered saline solution, containing (in mM): 137 NaCl, 5.4 KCl, 2 $CaCl_2$, 15 HEPES. Endothelial cells were removed using dispase (Boehringer Mannheim, type II) as described previously [10]. Subsequently, disaggregation of smooth muscle tissue was achieved by filling the vessels with low Ca^{2+} (0.1 mM) Hanks buffer (Sera Lab Ltd.) which was supplemented with 0.5 mg/ml collagenase (Worthington, type II), 0.5 mg/ml trypsin inhibitor (Worthington) and 1 mg/ml fatty acid-free bovine serum albumin. Vessels filled with collagenase containing Hanks solution, were incubated for 10 min at 37 °C. Thereafter the Hanks buffer which now contained single, mostly relaxed, elongated smooth muscle cells, was collected, centrifuged (5 min; 250 × *g*), and the cells were resuspended and stored in a solution containing [mM]: 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 HEPES, 2 EGTA (pCa = 7, see below) at 4°C, and used for experimentation within 36 hours.

2.2. Current measurements

Ba^{2+} currents through single Ca^{2+} channels were recorded in cell-attached patches of membrane. In order to establish constant cell po-

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Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethane-sulfonic acid; P_o, channel's probability of being in the open state; PKC, protein kinase C; PDD, 4-α-phorbol-12,13-didecanoate; TEA-Cl, tetraethylammonium chloride; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

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tentials of approximately 0 mV, we used a high K^+ low Cl^- extracellular solution which contained [mM]: 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 HEPES, 2 EGTA, pH was adjusted to 7.4 with *N*-methyl-D-glucamine, and pCa was adjusted according to Robertson and Potter [11]. The pipette solution contained [mM]: 80 $BaCl_2$, 30 TEA-Cl and 15 HEPES, pH adjusted to 7.4. Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments; Pangbourne UK), and had resistances of 5–10 MΩ. Experiments were performed at room temperature. Since basal channel activity was usually low, which impeded quantification of effects, the dihydropyridine-Ca^{2+} channel activator S(−)-BayK 8644 (0.5 μM) was added to the pipette solution in most experiments. For voltage clamp and current amplification, a List EPC/7 patch-clamp amplifier (List, Darmstadt, Germany) was used. Current records were stored on video tape using a VR10 digital data recorder (Instrutech Corporation, Mineola, USA). Records were played back, filtered at 1 kHz (−3dB) and digitized at a rate of 5 kHz. Data were analyzed with pClamp (Axon Instruments, Foster City, CA, USA) and Transit (BCM, Houston, TX, USA) software. Channel actvity in multichannel patches was calculated as the mean number of channels which opened during depolarizing voltage steps. The availability of a channel to open upon depolarization was quantified as the proportion of sweeps which showed at least one opening (active sweeps), according to the applied criteria of level detection (Transit software). Averaged data are presented as mean ± S.E.M.

2.3. Chemicals

Collagenase, type CLS II and soybean trypsin inhibitor type were obtained from Worthington Biochemical Corporation (Freehold, NJ, USA); dispase type II was from Boehringer (Mannheim, Germany); fatty acid-free bovine serum albumin from Behring (Marburg, Germany); Hanks balanced salt solution was from Sera Lab Ltd. (Sussex, UK); Chelerythrine was from LC Services Corp. (Woburn, MA, USA); S(−)-BayK 8644 and H-7 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine were purchased from Research Biochemicals Incorporated (Natic, MA, USA); TPA, PDD, and all other chemicals from Sigma Chemical Co. (Deisenhofen, Germany).

3. Results

3.1. TPA exerts a dual modulatory effect on smooth muscle Ca^{2+} channels

Using 80 mM Ba^{2+} as charge carrying cation, L-type Ca^{2+} channels in cell-attached patches of human umbilical vein exhibited a unitary conductance of approximately 21 pS (20.6 ± 0.5, $n = 17$) and typical voltage dependence showing a threshold of activation above −30 mV. Time courses of channel activity in cell-attached patches were monitored by depolarizing the patches of membrane every 5 s to 0 mV from a holding potential of −70 mV (referring to the cytoplasmic side). Exposure of the cells to TPA in concentrations up to 30 nM reduced channel activity in cell-attached patches within 1–3 min. A representative experiment demonstrating inhibition of channel activity by 10 nM TPA is illustrated in Fig. 1A. In contrast, one order of magnitude higher TPA concentrations (100 nM) induced a marked but transient increase of channel activity which was followed by sustained channel inhibition (Fig. 1B). These effects of TPA were observed in the presence and absence of 0.5 μM S(−)-BayK 8644 (included in the pipette solution) as well. Fig. 1C illustrates the effects of cumulative administration of TPA on channel activity recorded in the absence of S(−)-BayK 8644. When the TPA concentration was sequentially increased in the bath solution, L-

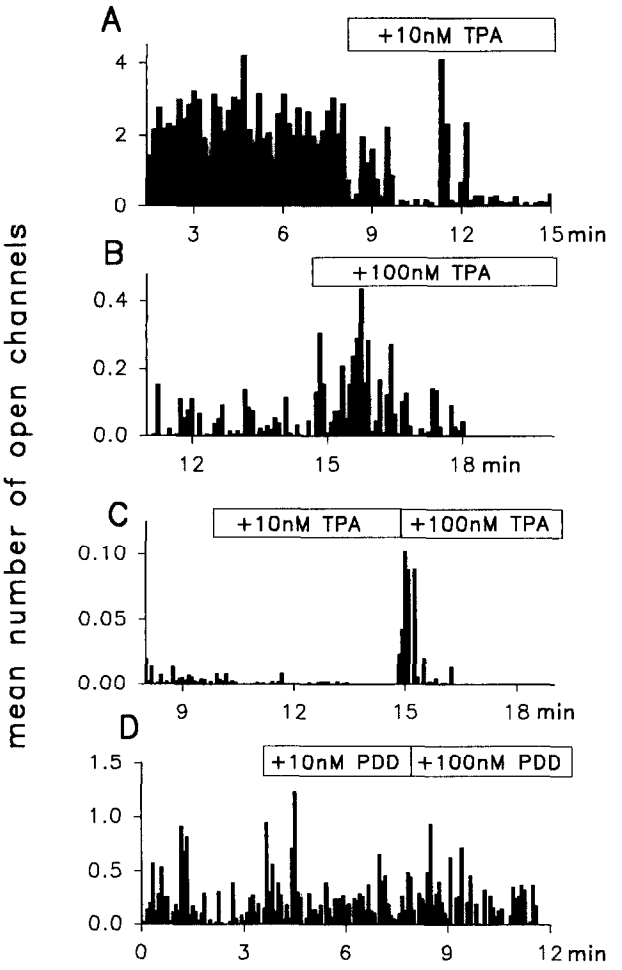

Fig. 1. Effects of phorbol ester on L-type Ca^{2+} channel activity in cell-attached patches. Depolarizing voltage steps (test potential = 0 mV, holding potential = −70 mV) were applied every 5 s and channel activity during individual steps is expressed as mean number of open channels. (A) Time course of channel activity during extracellular administration of 10 nM TPA. (B) Effect of 100 nM TPA. (C) Effects of sequential administration of 10 and 100 nM TPA. (D) Sequential administration of 10 and 100 nM PDD. Experiments illustrated in A,B and D were performed in the presence of 0.5 μM S(−)-BayK 8644 (included in the pipette solution).

type channels in cell-attached patches were first inhibited at 10 nM and subsequently stimulated at 100 nM. Fig. 1D shows an experiment in which the inactive 4-α-phorbol-12,13-didecanoate (PDD) was used instead of TPA. PDD neither inhibited nor stimulated L-type Ca^{2+} channel activity ($n = 3$). Fig. 2 shows the stimulatory effect of 100 nM TPA on channel activity recorded during depolarizing voltage ramps. TPA did not affect single channel conductance but apparently increased the number of channels which opend upon depolarization. During this transient stimulatory effect, voltage-dependence of activation was shifted to more negative potentials, as evident from the average current derived from 32 current responses to depolarizing voltage ramps (Fig. 2, lower panel). Fig. 3 summarizes the observed TPA-induced

modulation of channel activity, and in addition shows the effects of the PKC inhibitor H-7. Pretreatment of the cells with H-7 (10 μM for 5 min) which by itself slightly enhanced channel activity, abolished the inhibitory effect of 10 nM TPA and clearly suppressed the transient stimulatory effect of 100 nM TPA ($n = 4$). Consistantly, a similar result was obtained with the selective PKC inhibitor chelerythrine (1 μM, $n = 2$, not shown).

3.2. *TPA affects channel availability and open time distribution*

Experiments with patches of membrane containing a single L-type Ca^{2+} channel were analyzed for TPA-induced changes in channel availability and open time distribution. Fig. 4 illustrates an experiment in which no overlapping events were detected, indicating the presence of only one channel. At 10 nM TPA, the probability of the channel being in the open state (P_o) decreased from 27 to 16% (calculated from 25 and 30 voltage steps to 0 mV in the absence and presence of TPA respectively; Fig. 4 middle panel). This reduction of P_o was associated with a reduction of the channel's availability to open upon depolarization, i.e. the fraction of depolarizing voltage steps which exhibited channel activity (active sweeps; AS, Fig. 4, middle panel). In the presence of BayK 8644

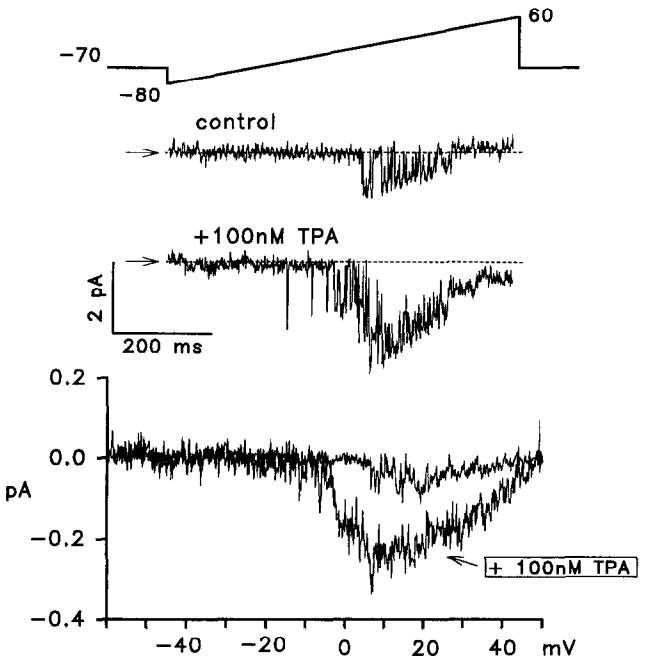

Fig. 2. Effect of TPA (100 nM) on current to voltage relationship. Channel activity was recorded during depolarizing voltage ramps (from −80 to +60 mV, 0.1 mV/ms, holding potential = −70 mV). Upper panel illustrates representative individual current responses recorded in the absence and presence of TPA. Leak current has been subtracted and zero current level is indicated by arrows and dashed lines. Lower panel shows the average currents obtained from 32 and 16 sweeps in the absence and presence of TPA respectively (0.5 μM S(−)-BayK 8644 was present in the pipette solution).

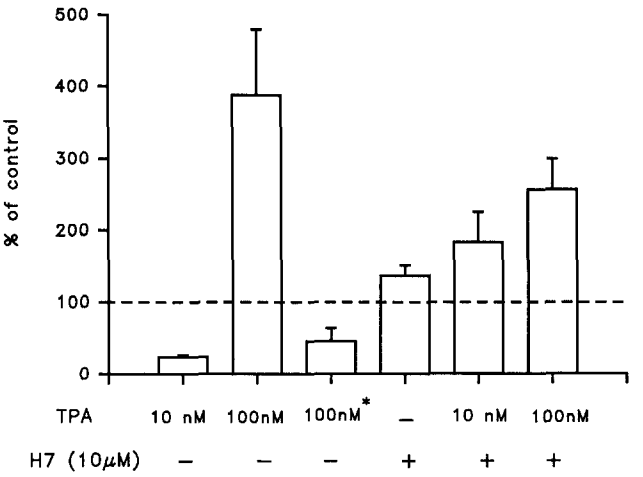

Fig. 3. Effects of TPA on L-type Ca^{2+} channel activity in the absence and presence of H-7. Channel activity was calculated from 30 s recordings (test potential = 0 mV) during the maximum responses (maximum deviation from control activity) induced by TPA, and expressed as% of control (pre-drug value). The effect of 100 nM TPA in the absence of H-7 was biphasic and is thus represented by two columns which show activity during transient stimulation and sustained inhibition (indicated by *, respectively. In all experiments S(−)-BayK 8644 (0.5 μM) was present in the pipette solution. Mean values ± S.E.M. are shown (n = 4–6).

(0.5 μM in the pipette solution), two open states ($\tau_{fast} = 0.58 \pm 0.14$ and $\tau_{slow} = 9 \pm 1.3$ ms, $n = 4$) were detected under control conditions. TPA (10 nM) clearly reduced the proportion of long openings (Fig. 4, lower panel). Although there was a moderate increase in τ_{slow}, changes in open time constants induced by 10 μM TPA were not statistically significant ($\tau_{fast} = 0.51 \pm 0.12$ and $\tau_{slow} = 14 \pm 4.5$ ms, $n = 4$; compare with control values above). Upon raising the TPA concentration to 100 nM, the channel's P_o increased transiently to 53%. Analysis of this transient effect of TPA (using 12 sweeps recorded during channel activation) showed an increase in the fraction of active sweeps (AS) to approximately 90% (middle panel) associated with a increase in the proportion of events which displayed long open times (lower panel). Although, analysis of open time distribution was impeded by the transient nature of the stimulatory effect of TPA, the data derived from current traces recorded during transient stimulation indicated that 100 nM TPA induced the occurrence of an extremely long lived open state ($\tau_{slow} = 78$ ms, mean = 62 ± 8 ms, $n = 5$). These results demonstrate a dual effect of TPA on P_o of smooth muscle L-type Ca^{2+} channels, which is based on a dual effect on channel availability and open time distribution.

4. Discussion

A large body of evidence suggests involvement of PKC in smooth muscle Ca^{2+} signalling [2–5,9], but the precise

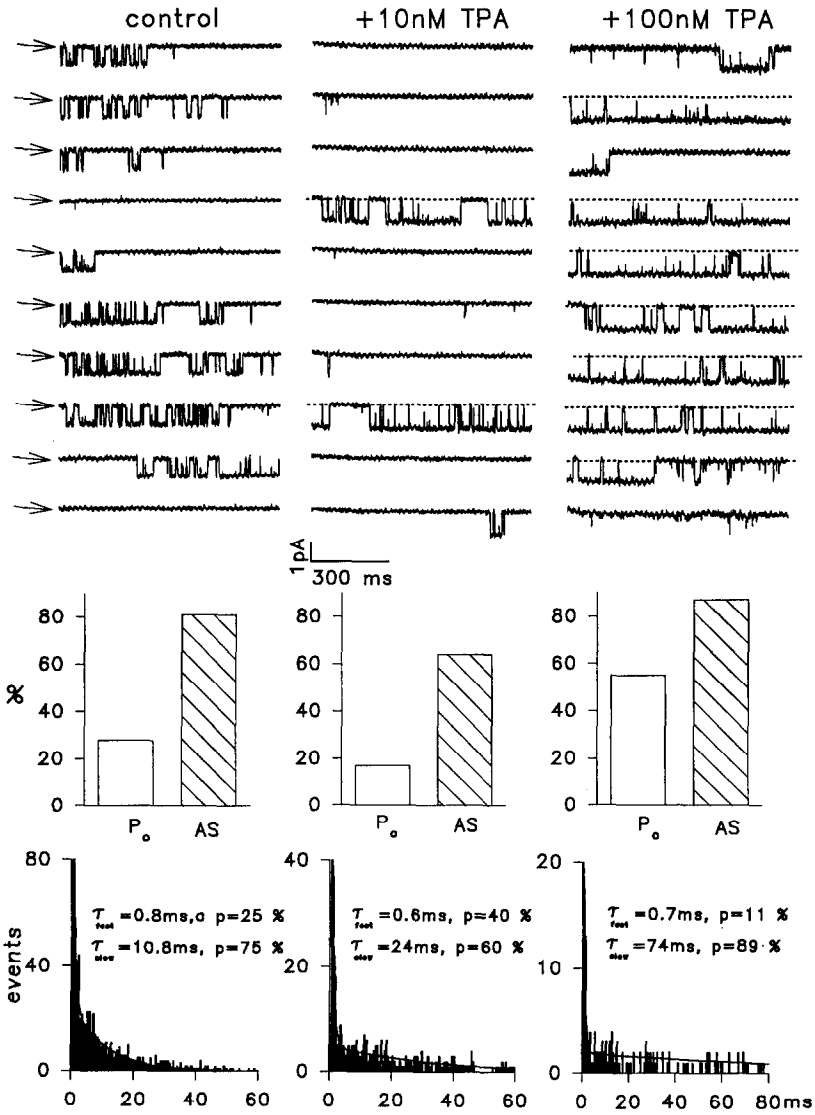

Fig. 4. TPA-induced modulation of open probability, availability and open time distribution of L-type Ca^{2+} channels. Upper panel illustrates representative current traces recorded during depolarizing voltage steps to 0 mV (holding potential = −70 mV) in the absence (control) and presence of 10 and 100 nM TPA. Closed state of the channel is indicated by arrows. Middle panel shows open probability (P_o) and availability (given as % active sweeps, AS), Lower panel illustrates open time histograms derived from 32 (control, 10 nM TPA) and 12 (100 nM TPA) sweeps. Channel open time constants (τ_{fast} and τ_{slow}) and probability distribution of corresponding events (p) are derived from bi-exponential fit of the data.

role of this enzyme family in Ca^{2+} handling by smooth muscle cells is far from being completely understood. In this paper we demonstrate that TPA-induced activation of endogenous PKC modulates L-type Ca^{2+} channels of human vascular smooth muscle cells in a dual manner. Stimulatory and inhibitory effects of TPA were observed, depending on the concentration of the phorbol ester and the time of exposure. Low concentrations of TPA i.e. 10–30 nM which represent approximately the EC_{50} value reported for inhibition of dihydropyridine-sensitive Ca^{2+} uptake in A7r5 cells [4] exerted a monophasic inhibitory effect, while high concentrations of TPA elicited a biphasic response which consisted of a transient stimulatory and a sustained inhibitory component of action. Biological responses induced by PKC activators are known to be complex in nature, and often comprise both fast and slow, long term components of action [12]. In cardiac muscle, 200 nM TPA was found to induce a biphasic change in L-type channel activity [6]. This finding is in accord with the observed biphasic effect of 100 nM TPA in smooth muscle. For cardiac Ca^{2+} channels, it has been suggested that PKC-dependent phosphorylation mediates channel activation, and that the biphasic response may reflect transient activation of PKC which is followed by sustained down-regulation of the enzyme. This may as well be taken as an explanation of the response observed with high concentrations of TPA in smooth muscle. It is however, unlikely that inhibition of Ca^{2+} channels by low TPA concentrations is also due to down-regulation of PKC, since (i) the effect

was not preceded by an initial stimulatory effect, (ii) subsequent application of a 10-fold higher concentration of TPA still induced transient channel activation, and (iii) the effect was not mimicked by protein kinase C inhibitors. TPA-induced stimulation and inhibition of smooth muscle L-type channels was antagonized by the protein kinase inhibitor H-7 and by chelerythrine which appears to be a highly selective inhibitor of PKC [13]. Moreover, the actions of TPA were not mimicked by the inactive phorbol ester PDD. It appears thus conceivable to conclude that the observed effects involve activation of PKC as the cellular target of TPA. Channel regulation may either involve phosphorylation of proteins constituting the Ca^{2+} channel complex or alternatively phosphorylation of other regulatory proteins within the smooth muscle cell. Both the α_1 and the β subunit of the skeletal muscle L-type Ca^{2+} channel have been demonstrated to serve as substrates for PKC in vitro [14,15], and reconstituted channels have been shown to be activated in response to in vitro phosphorylation [16]. It is therefore tempting to speculate about direct phosphorylation of channel proteins as the basis of the observed effects. The observed dual nature of phorbol ester-induced channel modulation in smooth muscle suggests the involvement of two phosphorylation sites in channel regulation. Activation of smooth muscle PKC by low concentrations of TPA resulted in a reduction of channel availability and a reduced proportion of long openings, while high concentrations increased channel availability as well as the proportion of long openings which was in addition associated with the occurrence of an extremely long lived open state. In vitro phosphorylation of skeletal muscle L-type Ca^{2+} channels has been reported to result mainly in an increase in the proportion of long openings associated with a moderate reduction in channel availability [16], while activation of PKC in cardiac cells has been shown to increase P_o of L-type channels due to increasing the number of openings during depolarizing pulses rather than mean open time [6]. These divergent results obtained in different muscle tissues may reflect tissue specific regulation of L-type channels which may well be based on tissue specific expression of regulatory proteins and/or PKC isoenzymes [12,17]. Although, the PKC isoenzymes expressed in human umbilical vein smooth muscle are as yet unknown, the coexistance of multiple PKC isoforms which differ with respect to cellular regulation and distribution [18] is likely. Whether TPA-induced dual modulation of smooth muscle Ca^{2+} channels involves differential activation of PKC isoforms remains to be clarified. Nonetheless, our finding of a dual phorbol ester-induced Ca^{2+} channel modulation in smooth muscle strongly suggests the existence of at least two distinct PKC-dependent mechanisms involving distinct phosphorylation sites at regulatory proteins. This dual regulation may provide an important mechanism responsible for fine tuning L-type Ca^{2+} channel function in smooth muscle.

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