Phorbol Ester Increases the Dihydropyridine-Sensitive Calcium Conductance in a Vascular Smooth Muscle Cell Line

R. David Fish, Giovanni Sperti, Wilson S. Colucci, and David E. Clapham

In vascular smooth muscle, phorbol esters cause a slowly developing contraction and an associated transmembrane calcium flux, both of which are inhibited by dihydropyridine calcium channel antagonists. In the A7r5 cultured vascular cell line, we used the whole-cell voltage-clamp technique to identify voltage-dependent calcium conductances and investigate the effect of phorbol esters on that conductance having characteristic dihydropyridine sensitivity (slowly inactivating, high-threshold, "L-type"). With barium as the charge carrier, large-amplitude (100–800 pA) inward currents of two types were characterized by their kinetics and voltage dependence. With holding potential — 80 mV, a rapidly inactivating, low-threshold current ("T-type") was activated by depolarizations above — 40 mV and was maximal at — 10 mV. With holding potential — 30 mV, this component was inactivated, and a second slowly inactivating, high-threshold current was activated above —10 mV and was maximal at +10 to +20 mV. These currents are similar to the T-type and L-type currents previously described in vascular smooth muscle cells. When added to the bath, the active phorbol ester, 12-O-tetradecanoyl phorbol-13-acetate (100 nM) increased the slowly inactivating (L-type) current by 32 ± 20% (n = 8, ±SD). Phorbol-12,13-dibutyrate (100 nM) caused a similar effect, but the inactive phorbol, 4-a-phorbol (100 nM), did not. We conclude that at least two distinct calcium conductances are expressed in A7r5 vascular smooth muscle cells, and that the dihydropyridine-sensitive calcium conductance is acutely modulated by phorbol esters, presumably acting through stimulation of protein kinase C. Such modulation may play a role in increasing transmembrane calcium influx mediated by agonist-receptor interactions that lead to activation of protein kinase C and may help to sustain or amplify calcium-dependent cell responses. (Circulation Research 1988;62:1049–1054)

In vascular smooth muscle flux studies, a number of agonists, including serotonin, acetylcholine, a-adrenergic agonists, and growth factors, have been shown to increase transmembrane calcium flux in association with contractile activity. In a variety of tissues, including vascular smooth muscle, agonist-receptor interactions result in an increase in phosphoinositide turnover and the generation of at least two putative second messengers, inositol trisphosphate (IP₃) and diacylglycerol (DAG) (reviewed in references 1 and 2). While IP₃ appears to mediate the rapid release of calcium from intracellular stores, DAG increases the activity of a calcium-dependent protein kinase,¹² and may be responsible for the sustained phase of cellular response in a number of cell types.³⁴ Protein kinase C can be stimulated directly by tumor-producing phorbol esters.⁸
phorbol ester, TPA, has been found to increase the average voltage-dependent calcium current as compared with untreated cells,14 an increase involving recruitment of covert calcium channels in a recent report.15 In contrast, in avian neurons, phorbol esters cause acute decreases in voltage-dependent calcium conductance.16 Therefore, we undertook to investigate possible electrophysiological effects of phorbol esters on the dihydropyridine-sensitive calcium conductance in A7r5 cells.

At least two types of calcium channels are present in nerve,17 heart,18 and smooth muscle,19-22 characterized by voltage dependence of activation and inactivation, kinetics, and response to organic and inorganic blockers. In the nomenclature of Tsien and coworkers,23 both T-type (transient, low-threshold) and L-type (long-lasting, high-threshold) calcium channels have been characterized in vascular smooth muscle.19-21 In rat mesenteric artery cells, Bean et al24 found both types of calcium current and showed that the L-type calcium current was the more sensitive to block by nitrendipine.

Using the whole-cell patch-clamp configuration in single A7r5 cells, we identified large, stable calcium currents of two types, similar to the T-type and L-type currents previously described. As in previous studies, the L-type current was blocked by the dihydropyridine, nitrendipine. We therefore used this model system to test the hypothesis that protein kinase C can acutely modulate the calcium current in vascular smooth muscle cells, by measuring the response of the peak L-type current in single cells to applications of 20 mM barium modified Ringer's solution. Under these conditions, large-amplitude inward calcium currents were elicited under physiological conditions (2 mM Ca-Ringer's bath solution) by depolarizing step potentials from a holding potential of -80 mV. Two kinetic components were evident, and the slowly inactivating current was preferentially increased when barium was used as the charge carrier. To study the slowly inactivating current, 20 mM Ba-modified Ringer's was used as the standard bath solution. Under these conditions, large-amplitude (100-800 pA) inward currents of two types were elicited and distinguished by their kinetics and voltage dependence of activation and inactivation. Figure 1 shows single-cell inward current records with successive 400-msec depolarizing test pulses at 3-second intervals from a holding potential of -80 mV (Figure 1A) and from a holding potential of -30 mV (Figure 1B). With holding potential -80 mV, a rapidly inactivating, low-threshold current was activated at -40 to -30 mV, maximal at -10 mV, with time to peak of 11 ± 1 msec (n = 4, ± SD) and half-time of decay of 14 ± 2 msec (n = 4, ± SD). This current is similar to the T-type current described in other preparations. A second slowly inactivating current, activated at a higher threshold, was also expressed with holding potential -80 mV. Both current components exhibited steady-state inactivation but with different ranges of voltage dependence. Thus, holding potentials of -30 mV completely inactivated the rapidly inactivating component, leaving the slow component expressed in isolation as shown in Figure 1B. This high-threshold, slowly inactivating current was activated at -20 to -10 mV, maximal at +10 to +20

Materials and Methods

Cell Culture and Preparation

The A7r5 rat aortic smooth muscle cell line6 was obtained from the American Type Culture Collection, Bethesda, Maryland. As previously described,14 cells were plated at an initial density of 5,000-9,000 cells/cm², and grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, New York) containing antibiotics and 10% fetal calf serum (GIBCO), in a humidified atmosphere (37°C) under 5% CO₂-95% 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/cm². Cells used for experiments were from passages 6 through 35, during which time they exhibited stable morphological characteristics by light microscopy and reproducible electrophysiological responses under whole-cell voltage-clamp. Immediately prior to each experiment, confluent cell layers were dispersed with trypsin solution containing 0.05% trypsin, 0.68 mM EDTA, in Hanks' medium (pH 7.4) for 7-10 minutes with periodic agitation, resuspended in Hanks' medium containing 10% fetal calf serum, and plated at a density of 5,000 cells/ml for use from 1 to 5 hours after plating.

Whole-Cell Patch-Clamp

Isolated cells were bath-perfused at 22°C with 20 mM Ba-modified Ringer's solution (NaCl 113 mM, KCl 4 mM, MgCl₂ 2 mM, BaCl₂ 20 mM, NaOH-HEPES 10 mM, pH 7.4) in the whole-cell patch-clamp configuration with cesium-containing solution (CsCl 200 mM, Cs-EGTA 10 mM, MgCl₂ 1.4 mM, HEPES 10 mM, pH 7.2) in the patch pipette. In addition, the pipette solution contained 3.6 mM MgATP (Sigma Chemical, St. Louis, Missouri), 1 mM GTP (Sigma), 14.2 mM phosphocreatine (Sigma), and 50 units/ml creatine phosphokinase (Sigma) to minimize "wash-out" of the channels. Test agents added to the bath included 1) 100 nM 4α-phorbol, 2) 100 nM TPA, 3) 100 nM PDBu, and 4) 1 μM nitrendipine. Phorbol esters were diluted 1:10,000 from a stock solution in DMSO and were obtained from Sigma. Nitrendipine was diluted 1:1,000 from stock solution in ethanol, and was generously supplied by Miles Pharmaceuticals, West Haven, Connecticut.

Results

Characterization of Basal Calcium Currents

In the whole-cell voltage-clamp configuration, inward calcium currents were elicited under physiological conditions (2 mM Ca-Ringer's bath solution) by depolarizing step potentials from a holding potential of -80 mV. Two kinetic components were evident, and the slowly inactivating current was preferentially increased when barium was used as the charge carrier. To study the slowly inactivating current, 20 mM Ba-modified Ringer's was used as the standard bath solution. Under these conditions, large-amplitude (100-800 pA) inward currents of two types were elicited and distinguished by their kinetics and voltage dependence of activation and inactivation. Figure 1 shows single-cell inward current records with successive 400-msec depolarizing test pulses at 3-second intervals from a holding potential of -80 mV (Figure 1A) and from a holding potential of -30 mV (Figure 1B). With holding potential -80 mV, a rapidly inactivating, low-threshold current was activated at -40 to -30 mV, maximal at -10 mV, with time to peak of 11 ± 1 msec (n = 4, ± SD) and half-time of decay of 14 ± 2 msec (n = 4, ± SD). This current is similar to the T-type current described in other preparations. A second slowly inactivating current, activated at a higher threshold, was also expressed with holding potential -80 mV. Both current components exhibited steady-state inactivation but with different ranges of voltage dependence. Thus, holding potentials of -30 mV completely inactivated the rapidly inactivating component, leaving the slow component expressed in isolation as shown in Figure 1B. This high-threshold, slowly inactivating current was activated at -20 to -10 mV, maximal at +10 to +20...
mV, with time to peak of 24 ± 2 msec (n = 7, ± SD), corresponding to the L-type current previously described. Rates of inactivation for this slow component were highly variable with half-times ranging from 150 msec to many seconds. The current-voltage relations for the two current components of Figures 1A and 1B is shown in Figure 1C. The fast current component was measured by subtracting the slow component, measured with holding potential −30 mV where it is minimally inactivated, from the sum of the two currents obtained at holding potential −80 mV. As shown in Figure 1C, depolarizing steps to −20 mV activated the fast current but were below the threshold for activating the slow current.

Multicell averages of steady-state inactivation curves for the fast (n = 8) and slow (n = 4) current components are plotted as a function of holding potential in Figure 2. The fast current was completely inactivated at a holding potential of −30 mV with a half-inactivation point of −50 mV. This fast component of the same cell of Figure 1 is also shown for comparison. Some cells expressed only slow current; the inactivation of the isolated slow current measured in these cells is also shown in Figure 2 with a half-inactivation point of approximately −18 mV.

**Effects of TPA and PDBu on Calcium Currents**

Using a holding potential of −30 mV to inactivate the fast current, the acute effects of phorbol ester, added by bath perfusion, on the isolated slow current were studied. Slow current was measured with a series of test potentials from 0 to +30 mV to define the peak of the current-voltage relation each time. As shown in Table 1, TPA (100 nM) increased the magnitude of the peak slow current (as compared with pre-TPA control) by 12% to 66% in eight cells with an average of 32 ± 20% (n = 8, ± SD). Although the increases by TPA are not large, they are convincing since in the absence of TPA,
Table 1. Increases of Peak Slow (L-Type) Current by TPA* (100 nM) in 20 mM Ba

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Control current density (pA/pF)</th>
<th>Augmented current density (pA/pF)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.2</td>
<td>9.2</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>6.6</td>
<td>8.1</td>
<td>23</td>
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<tr>
<td>3</td>
<td>10.6</td>
<td>17.7</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>2.2</td>
<td>3.2</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>6.6</td>
<td>7.8</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>5.8</td>
<td>8.9</td>
<td>54</td>
</tr>
<tr>
<td>7</td>
<td>7.9</td>
<td>9.4</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>7.2</td>
<td>9.5</td>
<td>32 ± 20</td>
</tr>
</tbody>
</table>

*12-0-Tetradecanoyl phorbol-13-acetate.
†pA/pF, cell current/cell capacitance.

All cells showed a steady "rundown" of current (by a minimum of 15% in 5 minutes under our conditions). The time course of the augmentation by TPA is shown for a typical cell in Figure 3. After an initial control period, TPA (100 nM) caused a relative increase in peak slow current while in the other cell shown, the inactive phorbol, 4-α-phorbol (100 nM), did not affect the course of slow rundown. (These increases were above control levels, i.e., increases are expressed relative to pre-phorbol current and not merely relative to the course of rundown.) In Figure 4, sequential application of 4-α-phorbol and TPA in the same cell shows that TPA caused an increase in the slow current after the inactive phorbol did not. PDBu, another active phorbol, also increased the peak slow current above the course of rundown. The addition of 1 μM nitrendipine to the bath abolished the current in cells previously stimulated by TPA (data not shown), confirming that the TPA-stimulated current was carried by slow, L-type, dihydropyridine-sensitive channels. The augmentation by TPA was not reversible, although rundown superimposed on augmentation complicated the measurement. TPA (100 nM) did not shift the voltage dependence of the L-type current by more than 10 mV negative. No significant change of whole-cell current kinetics was observed with any of the tested phorbol esters.

Discussion

Rasmussen et al,6,7 Danthuluri and Deth,1 and others22,24 have observed that phorbol esters cause a slowly developing contraction in vascular smooth muscle. Most,7-22 but not all,1 studies have suggested that TPA-induced vascular contraction is dependent on external calcium, since it is abolished or attenuated by the removal of external calcium. Forder et al7 showed that the dihydropyridine calcium antagonist, nitrendipine, causes a substantial reduction in the amplitude of the TPA-induced contraction, suggesting that a significant amount of the associated transmembrane calcium flux passes through a dihydropyridine-sensitive membrane pathway. Using the A7r5 cell line, Sperti and Colucci11 recently showed that TPA increased the initial rate of dihydropyridine-sensitive transmembrane 43Ca influx in monolayers. That study suggested that the dihydropyridine-sensitive (presumably L-type) voltage-dependent calcium channel was modulated by phorbol activation of protein kinase C. These results are qualitatively similar to those in a preliminary report by Rüegg.13 However, the recent study of Galizzi et al12 found that preexposure of A7r5 cells to phorbol esters for 10 minutes inhibited KCl- or Bay K 8644-stimulated 45Ca uptake. The apparently different

**Figure 3.** Augmentation of the slowly inactivating (L-type) barium current (measured with holding potential −30 mV) by TPA (100 nM) (△), as compared with no effect of 4-α-phorbol (100 nM) (○). Currents are peak values normalized by control current prior to addition of phorbol ester, I∞.

**Figure 4.** Effect on slowly inactivating (L-type) barium current (measured with holding potential −30 mV) of sequential application of 4-α-phorbol (100 nM) and TPA (100 nM) in the same A7r5 cell. Currents are peak values normalized by control current prior to the addition of phorbol ester, I∞.
effects of phorbol ester on calcium flux in these two studies may be due to 1) different time periods studied (initial calcium uptake rate versus uptake after 10 minutes of phorbol ester preexposure), 2) inhibitory effects shown only in cells that had activated influx, and 3) substantially higher phorbol ester concentrations used by Galizzi et al (i.e., IC50 = 25 nM versus < 3 nM in Sperti and Calucci). In both of these flux studies, it was not possible to discern the relative contributions of direct effects of protein kinase C activation on the calcium channels and their indirect activation due to effects leading to membrane depolarization. Although Galizzi et al12 reported vasopressin-induced decrease of calcium current in whole-cell voltage-clamp experiments, comparison of our results is uncertain since a full kinetic or voltage-dependence description of the currents was not presented and experimental conditions differed. We have used the whole-cell voltage-clamp technique to study the calcium currents in A7r5 cells. Using this approach in which membrane potential is controlled, we have shown that the L-type calcium conductance is increased within minutes after exposure to phorbol esters.

In the A7r5 rat aortic cell line, we have identified two distinct voltage-dependent calcium currents, which are similar to the T-type (low-threshold, rapidly inactivating) and L-type (high-threshold, slowly inactivating) calcium currents described in other vascular preparations, including rat mesenteric artery,21 rat vein,22 and the related A10 cell line.23 The L-type current in the A7r5 cells also exhibits the expected dihydropyridine sensitivity and selectively higher barium conductance previously observed. Under conditions designed to isolate the slow, L-type current, our data demonstrate that the active phorbol esters, TPA and PDBu, cause an acute increase in the peak whole-cell current when applied in the external bath. This effect did not require pretreatment of the cells with phorbol ester as in a previous study24 and was abolished by the dihydropyridine antagonist, nitrendipine. Further, the increases were measured relative to the pre-phorbol control levels shortly after establishing whole-cell patch-clamp, and therefore were increases beyond the possible reversal or reduction of the effects of rundown. While Armstrong and coworkers25 have shown that A-kinase will activate protein kinase C through phosphoinositide second-messenger systems, there is no data on the specific calcium currents activated (as by potassium chloride depolarization), but as technical limitations allow, this important information will aid the understanding of both flux and electrophysiological results. If T-type currents were decreased by phorbol ester, then flux studies in which both types of calcium current are activated (as by potassium chloride depolarization), but modulated in opposing directions, may well yield variable and apparently contradictory results. Therefore, electrophysiological studies are needed to unambiguously describe the responses of calcium channels to regulatory agents.

Our observations indicate that protein kinase C can modulate calcium current through voltage-dependent calcium channels. If these findings in A7r5 cells reflect events in vascular smooth muscle in situ, they raise the possibility that a protein kinase C-mediated calcium current is involved in the regulation of calcium homeostasis during vascular contraction. These findings further provide a mechanism by which hormones that activate protein kinase C through phosphoinositide hydrolysis may influence calcium influx.

Acknowledgment

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Key Words: phorbol ester, protein kinase C, calcium, calcium channel, vascular smooth muscle, A7r5
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