0026-895X/95/040842-06$3.00/0

MOLECULAR PHARMACOLOGY, 47:842–847 (1995).

Direct Block of Calcium Channels by Dioctanoylglycerol in Pregnant Rat Myometrial Cells

MASUMI KUSAKA and NICHOLAS SPERELAKIS

Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267

Received November 8, 1994; Accepted January 13, 1995

SUMMARY

The effect of 1,2-dioctanoyl-*sn*-glycerol (DiC8), a diacylglycerol and potent protein kinase C (PKC) activator, on the voltage-dependent slow (L-type) Ca^{2+} current [$I_{Ca(L)}$] was examined using whole-cell voltage clamp of myometrial cells freshly isolated from late pregnant rats. Bath application of DiC8 (25 μM) decreased $I_{Ca(L)}$ by 50.7 ± 2.4% (n = 22). The effect was reversible and dose dependent (IC_{50} of 15.3 μM). The effect of DiC8 was not reversed or prevented by application of calphostin C, a selective PKC inhibitor. In addition, 1-oleoyl-2-acetyl-*sn*-glycerol, another PKC activator, did not produce an inhibitory effect on $I_{Ca(L)}$, even at a concentration of 100 μM; $I_{Ca(L)}$ was actually slightly stimulated (10.6 ± 5.1%, n = 6). The steady state inactivation curve for $I_{Ca(L)}$ was shifted to the left by DiC8 application (by approximately 15 mV at 25 μM), whereas the activation curve was not affected; the shift should produce a voltage-dependent block. DiC8 decreased $I_{Ca(L)}$ progressively during repetitive step depolarizations (use-dependent block). We conclude from these results that (a) DiC8 inhibits $I_{Ca(L)}$ independently of PKC in uterine muscle cells and (b) DiC8 preferentially acts on the inactivated state and/or open state of the L-type Ca^{2+} channels. Therefore, caution must be exercised when studying PKC actions on ion channel regulation using DiC8 as a PKC activator.

PKC activation produced by DAG, which is formed through PI turnover, is known to be an important intracellular signal transduction system for diverse hormones and transmitters and plays important roles in various cell functions (1). In uterine smooth muscle, several hormones appear to exert their actions through PI metabolism and resulting PKC activation (and production of inositol phosphates). For example, oxytocin increases inosititol phosphate production and $[Ca^{2+}]_i$ (2–5). Prostaglandins (4, 6), carbachol (2, 6, 7), and endothelin (8) were also reported to increase PI turnover in myometrium. Although it is obvious that $[Ca^{2+}]_i$ increases produced by these hormones are an important factor for their uterotonic action, it remains unclear whether PKC activation is involved.

DAG analogs and phorbol esters are popularly used as potent PKC activators. In vascular smooth muscle cells, phorbol esters are known to elicit contractions (9, 10). Both DAG analogs and phorbol esters increase Ca^{2+} influx (11–13). In uterine smooth muscle, phorbol esters were reported to augment electrically induced or KCl-induced myometrial contractions during pregnancy (14, 15). We previously demonstrated that activation of PKC by a phorbol ester increases $I_{Ca(L)}$ in cells isolated from pregnant rat myometrium (16). These results suggest that PKC activation modulates Ca^{2+} channel activity and increases $[Ca^{2+}]_i$ in smooth muscles, including uterine smooth muscle cells.

To further study this effect, we used DiC8, which is a DAG analog and a potent PKC activator. However, we unexpectedly found that DiC8 dose-dependently reduces $I_{Ca(L)}$ in myometrial cells. This effect of DiC8 was independent of PKC activation. The results indicate that DiC8 directly inhibits Ca^{2+} channels, and therefore care should be exercised when this agent is used as a PKC activator.

Materials and Methods

Cell preparation. Freshly isolated, single, smooth muscle cells were prepared from the longitudinal layer of late-pregnant (18–19-day) rat (Sprague-Dawley; Zivic-Miller) uterus as described previously (17). The rats were anesthetized with CO_2 and decapitated. The distal part of the uterine horn was excised and the longitudinal layer was carefully isolated by removing the endometrium and circular muscle layer under a binocular microscope. The tissues were incubated in nominally calcium-free solution (140 mM NaCl, 6 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.35) for 20 min and then with 0.2–0.3% collagenase (Worthington Biochemical Co., Freehold, NJ) for 80 min at 37°. After digestion, the tissues were rinsed twice in fresh calcium-free solution and cut into small pieces. They were

This work was supported by Grant HL26170 from the National Institutes of Health.

ABBREVIATIONS: PKC, protein kinase C; DiC8, 1,2-dioctanoyl-*sn*-glycerol; DAG, diacylglycerol; PI, phosphatidylinositide; $I_{Ca(L)}$, slow (L-type) Ca^{2+} current; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; HP, holding potential; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

agitated in modified KB solution (18) (50 mM potassium glutamate, 40 mM KCl, 20 mM KOH, 20 mM KH_2PO_4, 3 mM $MgCl_2$, 10 mM glucose, 20 mM taurine, 10 mM HEPES, 1 mM EGTA, pH 7.4) and stored at 4°. Cells were used for experiments within 4 hr after cell dispersion. Most of the cells obtained were spindle-shaped, 50–200 μm long, and 20–30 μm wide, as reported previously (19).

Patch-clamp recording. Whole-cell voltage-clamp recording was carried out with a suction pipette and a patch-clamp amplifier (Axopatch-1D; Axon Instruments, Foster City, CA), using standard techniques (20). The patch electrodes (2–5 MΩ) were made from borosilicate glass capillary tubing (World Precision Instruments). The cell suspension was placed into a small chamber (0.2 ml) on the stage of an inverted microscope (TMD-Diaphoto; Nikon, Tokyo, Japan). To isolate the $I_{Ca(L)}$, the pipette was filled with high-Cs^+ solution (110 mM CsOH, 20 mM CsCl, 110 mM glutamic acid, 5.2 mM $MgCl_2$, 10 mM HEPES, 5 mM Na_2ATP, 10 mM EGTA, pH 7.2). The bath solution contained 140 mM tetraethylammonium chloride, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, and 3 mM 4-aminopyridine, pH 7.35. Series resistance was partly compensated electrically. Leak current and residual capacitive current were subtracted using the *P*/4 protocol. Current and voltage signals were filtered at 2 kHz, digitized with an analog/digital converter (TL-1; Axon Instruments), and analyzed on an IBM-AT personal computer using pCLAMP software (Axon Instruments). The membrane capacitance was determined from the current amplitude elicited in response to a hyperpolarizing voltage ramp pulse of 0.2 V/sec, from a HP of 0 mV, to avoid interference from any time-dependent ionic currents. All experiments were carried out at room temperature (22–24°).

Data are presented as means ± standard errors. The significance of differences between mean values was evaluated using Student's *t* test. A value of $p < 0.05$ was considered to be significant.

Drugs. All drugs used were obtained from Sigma Chemical Co. (St. Louis, MO). DiC8, OAG, staurosporine, and calphostin C were dissolved in dimethylsulfoxide for stock solutions, prepared in aliquots, and frozen until use. The final maximal concentration of dimethylsulfoxide was 0.2%, which had no effect on $I_{Ca(L)}$.

Results

The mean membrane capacitance of uterine smooth muscle cells was 118.2 ± 4.5 pF (n = 35) and was not affected by application of DiC8 or other drugs used in this study. Unless stated otherwise, the $I_{Ca(L)}$ was elicited by 300-msec depolarizing step pulses from a HP of −60 mV every 20 sec. The current was completely inhibited by 1 μM nifedipine (data not shown), and the kinetic properties, including the inactivation time course and current-voltage relationship (Fig. 1), were consistent with those we reported previously for $I_{Ca(L)}$ in uterine smooth muscle cells (17, 19).

Bath application of DiC8 (25 μM) reduced $I_{Ca(L)}$ by 50.7 ± 2.4% (n = 22). Fig. 1A shows superimposed current traces before (Fig. 1Aa) and 8.5 min after (Fig. 1Ab) DiC8 application. Note the marked inhibition of the peak inward current produced by DiC8. The current-voltage relationship for peak $I_{Ca(L)}$ was not significantly shifted by DiC8; there was no obvious change in voltages for threshold or peak currents (Fig. 1B).

Fig. 2 shows the time course of the effect of DiC8 in one representative experiment. $I_{Ca(L)}$ was evoked by depolarizing pulses to +10 mV (from a HP of −60 mV, every 20 sec), and peak current amplitude was measured. $I_{Ca(L)}$ decreased gradually and reached a stable level within 10 min. The effect of DiC8 on $I_{Ca(L)}$ was reversed by washout, and a second addition of DiC8 again produced marked inhibition (Fig. 2).

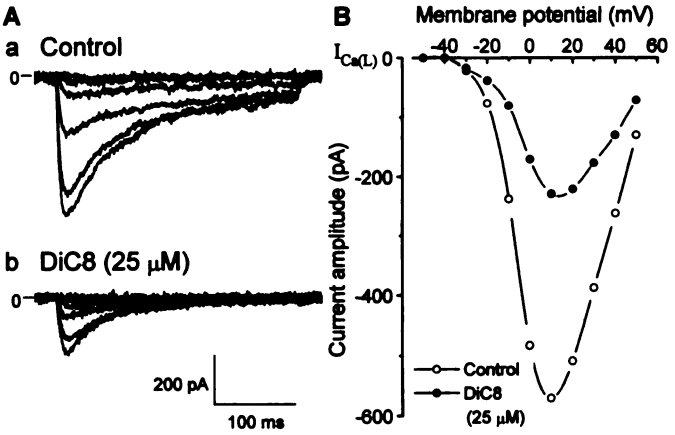

Fig. 1. Effect of DiC8 on $I_{Ca(L)}$ in 19-day pregnant rat uterine smooth muscle cells. A, Superimposed current traces elicited by 300-msec depolarizing pulses (−50 mV to +10 mV in 10-mV increments) from a HP of −60 mV, before (a) and 8.5 min after (b) bath application of 25 μM DiC8. B, Current-voltage relationships for $I_{Ca(L)}$ obtained before (○) and 8.5 min after (●) DiC8 application. The pipette solution contained Cs^+ to inhibit K^+ current and the bath solution contained 2 mM Ca^{2+}.

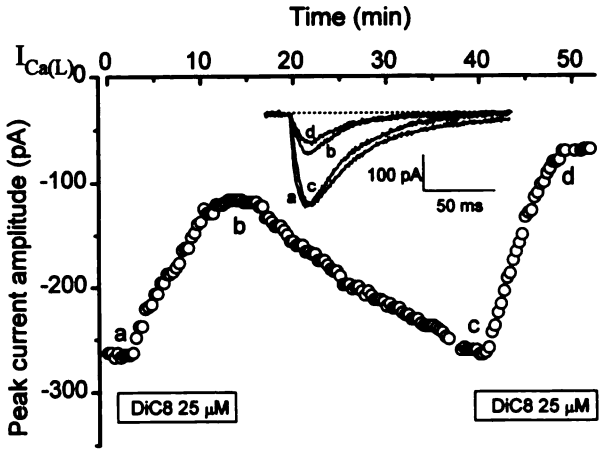

Fig. 2. Time course of the inhibition of $I_{Ca(L)}$ by 25 μM DiC8. The effect reached a stable level within 10 min after application of DiC8, was reversed by washout, and could be repeated. Currents were elicited from a HP of −60 mV to a step potential of +10 mV every 20 sec. *Inset*, superimposed current traces showing the $I_{Ca(L)}$ recorded before (*a*) and 12 min after (*b*) the first application of DiC8, after washout (*c*), and during the second application of DiC8 (*d*).

Washout of the effect was not complete when DiC8 was applied for a long period (data not shown).

Fig. 3 shows the dose-response curve for the inhibitory effect of DiC8. Data points were fitted to the Hill equation, $I_{DiC8}/I_{control}$ (%) = $E_{max}/[1 + ([DiC8]/IC_{50})^{n_H}] + (100 - E_{max})$, where E_{max} is the maximal inhibitory effect, n_H is the Hill coefficient, and IC_{50} is the half-inhibitory concentration of the drug. The n_H value was 1.43 and the IC_{50} value was 15.3 μM. The maximal inhibitory effect was calculated to be 74.8% and was almost attained at the concentration of 100 μM (70.0 ± 2.0%, n = 5).

To investigate the effects of DiC8 on the voltage dependence of activation and inactivation, steady state activation and inactivation (h_∞) curves were obtained before and after DiC8 application (Fig. 4). Activation curves were derived from the current-voltage relationships (Fig. 4, *right inset*). Conductance (G) was calculated from the equation $G = I_{Ca}/(V_m - V_{rev})$, where I_{Ca} is the peak current elicited by depo-

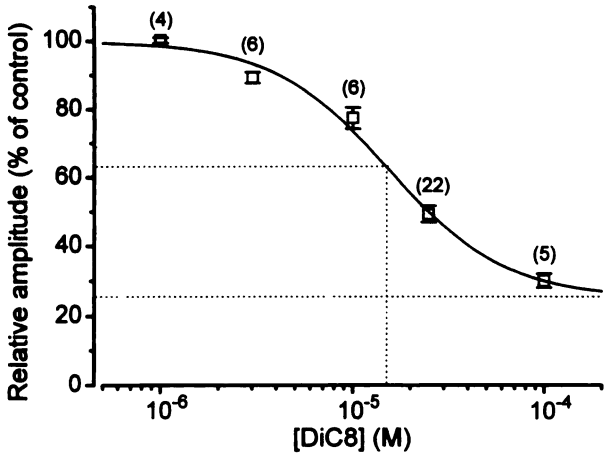

Fig. 3. Dose-response relationship for DiC8 inhibition of $I_{Ca(L)}$. *Numbers in parentheses*, number of cells for each data point. The IC_{50} and Hill coefficient (n_H) were 15.3 μM and 1.43, respectively.

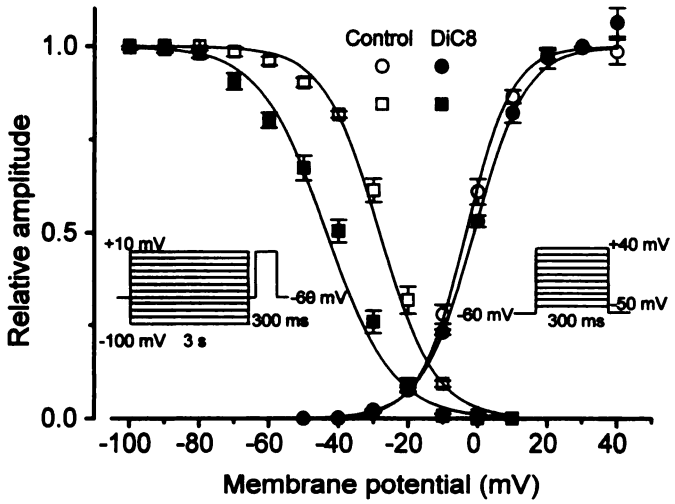

Fig. 4. Activation and inactivation curves for $I_{Ca(L)}$ before and after DiC8 (25 μM) application. Steady state inactivation (h_∞) curves were obtained by a double-pulse protocol. Preconditioning pulses of 3-sec duration were applied in 10-mV steps between −100 mV and +10 mV, from a HP of −60 mV, and then the test pulse of 300-msec duration was applied to +10 mV (interpulse duration, 5 msec). The inactivation curve in the presence of DiC8 was shifted toward a negative potential by about 15 mV, compared with the control. Activation curves were obtained from the current voltage relationships, and the voltage dependence was not changed by DiC8. *Insets*, pulse protocols; protocols and experimental conditions were the same as in Fig. 2.

larizing test pulses to the various potentials and V_{rev} is the reversal potential (+60.6 ± 1.3 mV, n = 18; obtained from the extrapolated current-voltage curves) (Fig. 1). G_{max} is the maximal Ca^{2+} conductance (calculated at potentials above +10 mV). The points for G/G_{max} were plotted against the membrane potential as a relative amplitude. These data were fitted to the Boltzmann equation, $1/[1 + \exp[(V_m - V_{1/2})/k]]$, where V_m is the conditioning potential, $V_{1/2}$ is the potential required for half-activation of current, and k is the slope factor. $V_{1/2}$ and k were −3.3 mV and −6.5 mV in controls and −1.1 mV and −7.4 mV (n = 7) after DiC8 application, respectively. There is no apparent significant difference among these values.

The h_∞ curves were obtained with a double-pulse protocol (Fig. 4, *left inset*). The peak current elicited by test pulses was normalized to the maximal current (evoked by a prepulse from −100 mV) and plotted against the conditioning potential. Resulting inactivation curves were also fitted to the Boltzmann equation. The values of $V_{1/2}$ (half-inhibition potential) and k were −28.2 mV and 8.0 mV in controls and −43.4 mV and 9.5 mV (n = 5) in the presence of DiC8, respectively. The inactivation curve was shifted in the negative direction by 15.2 mV, thereby indicating that DiC8 has a voltage-dependent inhibitory action.

To examine the possible use-dependent inhibition by DiC8 of $I_{Ca(L)}$, a train of test pulses (−60 mV to +10 mV for 40 msec) were applied at different pulse intervals, ranging from 0.25 to 5 sec (Fig. 5). In the absence of DiC8, $I_{Ca(L)}$ remained stable at 5-sec intervals, but when a pulse interval of 2 sec or shorter was applied there was progressive inhibition of current amplitude during the repetitive stimulation (Fig. 5A). Recovery to the initial current amplitude occurred within 1 min after cessation of the train of stimulation. In the presence of 25 μM DiC8, an additional decrease in $I_{Ca(L)}$ was observed even with stimulation at 5-sec intervals; the inhibition increased with each pulse and the blockage was cumulative (Fig. 5B). This effect of DiC8, in which an increase in the rate of stimulation facilitates the inhibition of the current, indicates use-dependent block.

To examine whether the effect of DiC8 on $I_{Ca(L)}$ was medi-

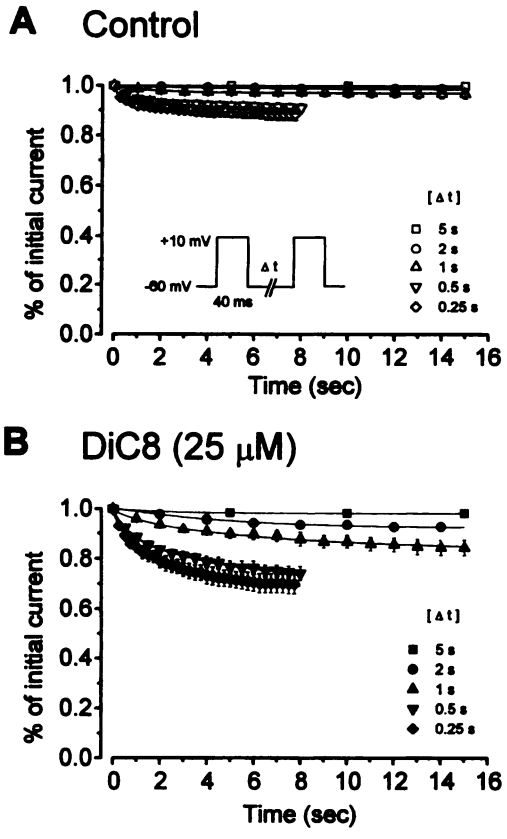

Fig. 5. Use-dependent inhibition by DiC8 of $I_{Ca(L)}$. The development of use-dependent inhibition of $I_{Ca(L)}$ in the presence of DiC8 is shown. The peak current amplitude of $I_{Ca(L)}$ was measured during repetitive stimulation at intervals (Δt) of 5, 2, 1, 0.5, and 0.25 sec, before (A) and 10 min after (B) bath application of DiC8 (25 μM). The peak current amplitudes were normalized to that induced by the first step pulse. *Inset*, pulse protocol. Each *point* is the mean ± standard error of five cells.

ated by PKC, experiments were performed using PKC inhibitors. The specific PKC inhibitor calphostin C (0.5 μM), when added to the bath after the DiC8 effect became stable, did not reverse the effect of DiC8 (n = 3) (Fig. 6A). In addition, even when cells were pretreated with calphostin C (0.5 μM) for 5–15 min, DiC8 could still reduce the current (Fig. 6B). This type of experiment was performed in four cells. We observed that 0.5 μM calphostin C could reverse the enhancement of $I_{Ca(L)}$ produced by a phorbol ester.[1] Staurosporine (0.5 μM), a potent inhibitor of protein kinases including PKC, also did not reverse the inhibition of $I_{Ca(L)}$ produced by DiC8 (data not shown).

We also tested the effect of OAG, another DAG analog and PKC activator, on $I_{Ca(L)}$. Bath application of OAG (25 μM) actually slightly increased $I_{Ca(L)}$, by 10.6 ± 5.1% (n = 6), rather than reducing the current. OAG did not inhibit $I_{Ca(L)}$ even at a concentration of 100 μM for up to 10 min. Subsequent application of DiC8 (100 μM) after washout of OAG markedly reduced the current (Fig. 7).

Discussion

In the present study, we found that DiC8 dose-dependently inhibits $I_{Ca(L)}$ in freshly isolated, uterine smooth muscle cells from late-pregnant rats. DiC8 shifts the inactivation curve for $I_{Ca(L)}$ in the negative direction and produces use-dependent inhibition. The effect of DiC8 was not affected by PKC inhibitors, and another DAG analog, OAG, did not produce a decrease of the current, indicating that $I_{Ca(L)}$ inhibition by DiC8 is independent of PKC activation.

DAG analogs are widely used to study the effect of PKC on various cell functions, including modulation of $I_{Ca(L)}$. For example, in myocardial cells OAG was reported to increase

[1] M. Kusaka and N. Sperelakis, unpublished observations.

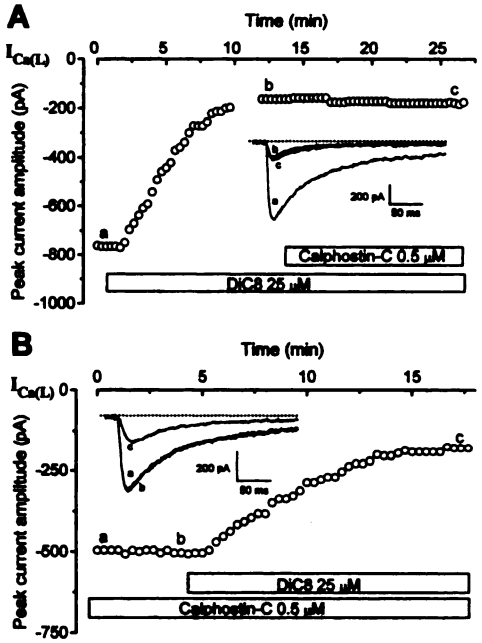

Fig. 6. Lack of prevention or reversal of DiC8 effects by the PKC inhibitor calphostin C. A, Calphostin C (0.5 μM) did not reverse the inhibition of $I_{Ca(L)}$ produced by DiC8 (25 μM). B, Pretreatment with calphostin C for 5 min did not prevent the effect of DiC8. Experimental conditions were the same as in Fig. 2.

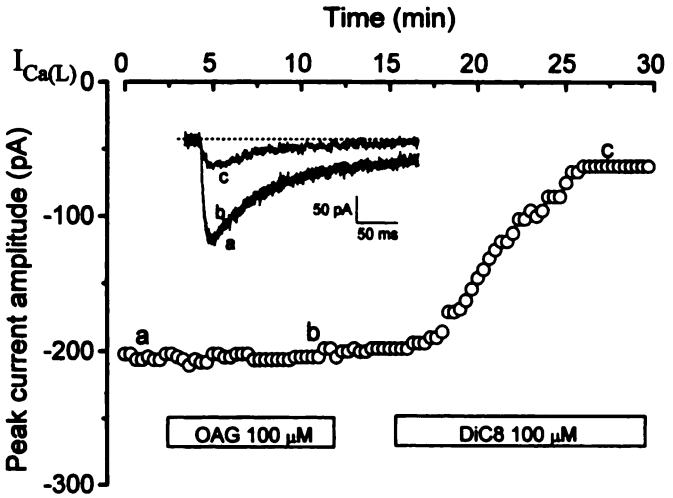

Fig. 7. Lack of effect of OAG, another DAG analog and a PKC activator, on $I_{Ca(L)}$. Experimental conditions were the same as in Fig. 2. Bath application of OAG (100 μM) did not inhibit $I_{Ca(L)}$ up to 10 min. Subsequent application of DiC8 (100 μM) markedly reduced the current. *Inset*, superimposed current traces recorded during the control period (*a*) and in the presence of OAG (*b*) and DiC8 (*c*).

$I_{Ca(L)}$ (21, 22), whereas in neuronal cells OAG was reported to reduce $I_{Ca(L)}$ (23–26). PKC activators, including DiC8, were reported to inhibit Ca^{2+} channel activity in GH_3 pituitary cells (27). In contrast, in neuronal cells DiC8 was reported to increase Ca^{2+} influx through L-type Ca^{2+} channels (28) and to recruit silent (covert) Ca^{2+} channels with higher single-channel conductance (29). For smooth muscle cells, it was reported that DiC8 enhances $I_{Ca(L)}$ in myocytes of toad stomach (12) but inhibits Ca^{2+} channel activity in the A7r5 cell line derived from rat aortic cells (30). In all of these reports, it was considered that the effects of these agents are mediated by PKC activation. One possible explanation for the differences in the effects on $I_{Ca(L)}$ produced by DAG analogs is the diversity of the tissues used, and DAG analogs could activate different subtypes of PKC in the different tissues.

In the present study, we showed that DiC8 directly inhibits $I_{Ca(L)}$, independently of PKC, in myometrial cells from late-pregnant rats. The concentration range of DiC8 that affected $I_{Ca(L)}$ (IC_{50} = 15.3 μM) is similar to that used in previous studies and is comparable to the concentration needed for PKC activation. Therefore, this direct effect of DiC8 on Ca^{2+} channels may contribute to the discrepancy in the results from the previous reports described above. Consistent with our present findings, several PKC activators, including DiC8, were reported to reduce $I_{Ca(L)}$ independently of PKC activation in neuronal cells (31). However, 5 μM OAG was sufficient to give about 58% inhibition of $I_{Ca(L)}$, whereas DiC8 (50 μM) and phorbol esters reduced the current by only 18%. As discussed below, the inhibitory effect of DiC8 is dependent on the conformational state of the channels; thus, the different pulse protocols used in the two studies could affect the results.

DiC8 shifts the steady state inactivation curve to the left, indicating that the effect of DiC8 is voltage dependent and therefore should be more prominent when the membrane is held at depolarized potentials (Fig. 4). Increases in the stimulation rate accentuated the inhibition of $I_{Ca(L)}$ produced by DiC8. This phenomenon is well known as use or frequency

dependence, similar to the effects of local anesthetics on Na^+ channels (32) and of most Ca^{2+} antagonist drugs on Ca^{2+} channels (33). The leftward shift of the steady state inactivation curve suggests that DiC8 binds more strongly to the inactivated state than to the resting state of the Ca^{2+} channels, and the use dependence indicates that DiC8 prefers the open state and/or the inactivated state. Therefore, these results suggest that DiC8 acts directly on the L-type Ca^{2+} channels and preferentially binds to the open and/or inactivated states of the channels in uterine muscle cells.

OAG (25 μM) increased $I_{Ca(L)}$ by only 11%. We previously reported, using the nystatin-perforated patch, whole-cell, voltage-clamp technique to maintain the cytosol components, that a phorbol ester (phorbol-12,13-dibutyrate) enhanced $I_{Ca(L)}$ by 34% through PKC activation in myometrial cells (16). In the present study, using the conventional whole-cell clamp technique, the small stimulatory effect of OAG (11%) may be due to possible loss of substances needed for optimal activation of PKC. Furthermore, the phorbol esters enhanced $I_{Ca(L)}$ only at high Ca^{2+} concentrations (in the pipette solution) when the conventional whole-cell clamp technique was used.[2] In vascular smooth muscle cells, a phorbol ester was reported to activate the nonselective cation channel only when guanosine-5′-*O*-(3-thio)triphosphate was present in the pipette (34). The strong direct inhibitory effect of DiC8 could mask a relatively small stimulatory effect via PKC activation. If OAG, at 100 μM, also exerted a small inhibitory effect on $I_{Ca(L)}$, it could cancel a small stimulation of the current. It is also possible that the DAG analogs and phorbol esters may activate different subtypes of PKC that have diverse effects on Ca^{2+} channels. For example, it was reported that the depolarization-induced Ca^{2+} influx in neuronal cells could be differentially modulated by DiC8 and the phorbol esters (28).

There are some other possible explanations for the inhibitory effect of DiC8. Possible changes in cell surface area (capacitance) could affect $I_{Ca(L)}$; however, in our study, the cell capacitance was not different before and after DiC8 application. Phorbol esters were reported to inhibit oxytocin-stimulated contraction in nonpregnant rat uterine muscle by causing rapid down-regulation of PKC (35). However, this effect has not been shown for DAG analogs, and inhibition of $I_{Ca(L)}$ occurred within a few minutes after application of DiC8.

In conclusion, DiC8 inhibits $I_{Ca(L)}$ in uterine smooth muscle cells by directly acting on the channels. This effect is not mediated by PKC activation and is voltage and use dependent. Because the concentration range for producing the inhibitory effect is comparable to that needed for activation of PKC, care should be taken in studies of PKC action using DiC8 as a PKC activator.

References

1. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.)* **308**:693–698 (1984).
2. Anwer, K., and B. M. Sanborn. Changes in intracellular free calcium in isolated myometrial cells: role of extracellular calcium and possible involvement of guanine nucleotide-sensitive proteins. *Endocrinology* **124**: 17–23 (1989).
3. Marc, S., D. Leiber, and S. Harborn. Fluoroaluminates mimic muscarinic- and oxytocin-receptor-mediated generation of inositol phosphates and contraction in the intact guinea pig myometrium: role for a pertussis/cholera toxin-insensitive G protein. *Biochem. J.* **255**:705–711 (1988).
4. Molner, M., and F. Hertelendy. Regulation of intracellular free calcium in human myometrial cells by prostaglandin $F_{2\alpha}$: comparison with oxytocin. *J. Clin. Endocrinol. Metab.* **71**:1243–1250 (1990).
5. Schrey, M. P., P. A. Cornford, A. M. Read, and P. J. Steer. A role for phosphoinositide hydrolysis in human uterine smooth muscle during parturition. *Am. J. Obstet. Gynecol.* **159**:964–970 (1988).
6. Ruzycky, A. L., and D. J. Crankshaw. Role of inositol phospholipid hydrolysis in the initiation of agonist-induced contraction of rat uterus: effects of domination by 17-estradiol and progesterone. *Can. J. Physiol. Pharmacol.* **66**:10–17 (1988).
7. Varol, F. G., M. Hadjiconstantinou, F. P. Zuspan, and N. H. Neff. Pharmacological characterization of the muscarinic receptors mediating phosphoinositide hydrolysis in rat myometrium. *J. Pharmacol. Exp. Ther.* **249**:11–15 (1989).
8. Khac, L. D., S. Naze, and S. Harbon. Endothelin receptor type A signals both the accumulation of inositol phosphates and the inhibition of cyclic AMP generation in rat myometrium: stimulation and desensitization. *Mol. Pharmacol.* **46**:485–494 (1994).
9. Danthuluri, N. R., and R. C. Deth. Phorbol ester-induced contraction of arterial smooth muscle and inhibition of α-adrenergic response. *Biochem. Biophys. Res. Commun.* **125**:1103–1109 (1984).
10. Rasmussen, H., J. Forder, I. Kojima, and A. Scriabine. TPA-induced contraction of isolated rabbit vascular smooth muscle. *Biochem. Biophys. Res. Commun.* **122**:776–784 (1984).
11. Chiu, P. J. S., G. Tetzloff, M. Chatterjee, and E. J. Sybertz. Phorbol 12,13-dibutyrate, an activator of protein kinase C, stimulates both contraction and Ca^{2+} fluxes in dog saphenous vein. *Naunyn-Schmiedebergs Arch. Pharmacol.* **338**:114–120 (1988).
12. Fish, R. D., G. F. Sperti, W. S. Colucci, and D. E. Clapham. Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. *Circ. Res.* **62**:1049–1054 (1988).
13. Vivaudou, M. B., L. H. Clapp, J. V. Walsh, Jr., and J. J. Singer. Regulation of one type of Ca^{2+} current in smooth muscle cells by diacylglycerol and acetylcholine. *FASEB J.* **2**:2497–2504 (1988).
14. Missiaen, L., Y. Kanmura, J. A. Eggermont, and R. Casteels. TPA- and agonist-induced force development in myometrium from pregnant and non-pregnant rats. *Biochem. Biophys. Res. Commun.* **158**:302–306 (1989).
15. Savineau, J.-P., and J. Mironneau. An analysis of the action of phorbol 12,13-dibutyrate on mechanical activity in rat uterine smooth muscle. *J. Pharmacol. Exp. Ther.* **255**:133–139 (1990).
16. Shimamura, K., M. Kusaka, and N. Sperelakis. Protein kinase C stimulates Ca^{2+} current in pregnant rat myometrial cells. *Can. J. Physiol. Pharmacol.* **72**:1304–1307 (1994).
17. Ohya, Y., and N. Sperelakis. Fast Na^+ and slow Ca^{2+} channels in single uterine smooth muscle cells from pregnant rats. *Am. J. Physiol.* **257**:C408–C412 (1989).
18. Isenberg, G., and V. Klockner. Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium." *Pfluegers Arch.* **395**:6–18 (1982).
19. Inoue, Y., and N. Sperelakis. Gestational change in Na^+ and Ca^{2+} channel current densities in rat myometrial smooth muscle cells. *Am. J. Physiol.* **260**:C658–C663 (1991).
20. Hamill, O. P., A. Marty, E. Nehr, B. Sakman, and F. S. Sigsworth. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:83–100 (1981).
21. Bourinet, E., F. Fournier, P. Lory, P. Charnet, and J. Nargeot. Protein kinase C regulation of cardiac calcium channels expressed in *Xenopus* oocytes. *Pfluegers Arch.* **421**:247–255 (1992).
22. Liu, Q.-Y., E. Karpinski, and P. K. T. Pang. Comparison of the action of two protein kinase C activators on dihydropyridine-sensitive Ca^{2+} channels in neonatal rat ventricular myocytes. *Biochem. Biophys. Res. Commun.* **191**: 796–801 (1993).
23. Doerner, D., M. Abdel-Latiff, T. B. Rogers, and B. E. Alger. Protein kinase C-dependent and -independent effects of phorbol esters on hippocampal calcium channel current. *J. Neurosci.* **10**:1699–1706 (1990).
24. Lewis, D, and F. F. Weight. The protein kinase C activator 1-oleoyl-2-acetylglycerol inhibits voltage-dependent Ca^{2+} current in the pituitary cell line AtT-20. *Neuroendocrinology* **47**:169–175 (1988).
25. Marchetti, C., and A. M. Brown. Protein kinase activator 1-oleoyl-2-acetyl-*sn*-glycerol inhibits two types of calcium currents in GH_3 cells. *Am. J. Physiol.* **254**:C206–C210 (1988).
26. Rane, S., and K. Dunlap. Kinase C activator 1,2-oleoylacetylglycerol attenuates voltage-dependent calcium current in sensory neurons. *Proc. Natl. Acad. Sci. USA* **83**:184–188 (1986).
27. Haymes, A. A., Y. W. Kwan, J. P. Arena, R. S. Kass, and P. M. Hinkle. Activation of protein kinase C reduces L-type calcium channel activity of GH_3 pituitary cells. *Am. J. Physiol.* **263**:C1211–C1219 (1992).
28. MacEwan, D. J., and R. Mitchell. Calcium influx through 'L'-type channels into rat anterior pituitary cells can be modulated in two ways by protein kinase C (PKC-isoform selectivity of 1,2-dioctanoyl-*sn*-glycerol?). *FEBS Lett.* **291**:79–83 (1991).
29. Strong, J. A., A. P. Fox, R. W. Tsien, and L. K. Kaczmarek. Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature (Lond.)* **325**:714–717 (1987).
30. Galizzi, J.-P., J. Qar, M. Fosset, C. V. Renterghem, and M. Lazdunski.

[2] M. Kusaka and N. Sperelakis, unpublished observations.

Regulation of calcium channels in aortic muscle cells by protein kinase C activators (diacylglycerol and phorbol esters) and by peptides (vasopressin and bombesin) that stimulate phosphoinositide breakdown. *J. Biol. Chem.* **262**:6947–6950 (1987).
31. Hockberger, P., M. Toselli, D. Swandulla, and H. D. Lux. A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. *Nature (Lond.)* **338**:340–342 (1989).
32. Courtney, K. R. Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by lidocaine derivative GEA 968. *J. Pharmacol. Exp. Ther.* **195**:225–236 (1975).
33. Kuga, T., J. Sadoshima, H. Tomoike, H. Kanaide, N. Akaike, and M. Nakamura. Actions of Ca^{2+} antagonists on two types of Ca^{2+} channels in rat aorta smooth muscle cells in primary culture. *Circ. Res.* **67**:469–480 (1990).
34. Oike, M., K. Kitamura, and H. Kuriyama. Protein kinase C activates the non-selective cation channel in the rabbit portal vein. *Pfluegers Arch.* **424**:459–464 (1993).
35. Phillippe, M. Protein kinase C, an inhibitor of oxytocin-stimulated phasic myometrial contractions. *Biol. Reprod.* **50**:855–859 (1994).

Send reprint requests to: Masumi Kusaka, Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, P.O. Box 670576, Cincinnati, OH 45267–0576.