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Effect of Protein Kinase C on Endoplasmic Reticulum Cholesterol

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Plasma membrane cholesterol both regulates and is regulated by effector proteins in the endoplasmic reticulum (ER) through a feedback system that is poorly understood. We now show that ER cholesterol varies over a fivefold range in response to experimental agents that act upon protein kinase C (PKC). Agents that activate Ca^{2+}-dependent PKC [phorbol-12-myristate-13-acetate (PMA) and bryostatin 1] increased the level of ER cholesterol; inhibitors such as staurosporine and calphostin C decreased it. Rottlerin, a selective inhibitor of the PKC-δ isoform, also increased ER cholesterol. The esterification of plasma membrane cholesterol was altered by protein kinase C-directed agents in a corresponding fashion. Furthermore, the regulatory effect of plasma membrane cholesterol on the esterification of ER cholesterol was blocked by PKC-directed agents. These findings suggest that multiple protein kinase C isoforms participate in the regulation of ER cholesterol and therefore in cholesterol homeostasis.

Key Words: **cholesterol homeostasis; protein kinase C; endoplasmic reticulum cholesterol.**

The sterols in eukaryotic cells are dynamically and nonuniformly distributed among endomembrane compartments in a complex and regulated fashion (1, 2). Approximately 85% of the cholesterol in human fibroblasts is located at the cell surface (3, 4) and ~5% in lysosomes (5); most of the remainder appears to be in endosomes (1, 2) and the Golgi apparatus (6).

The ER in fibroblasts typically contains only ~0.5% of the cell cholesterol (7, 8). Yet it is this compartment that bears the principal effectors governing cell cholesterol homeostasis. These include acyl-CoA:cholesterol acyl transferase and hydroxymethylglutaryl-CoA reductase as well as SREBP and SCAP, a transcriptional activator system for effectors of sterol accumulation (9). Each of these ER elements is under feedback control by cell cholesterol (10, 11). It appears that the ER pool is set according to the needs of the plasma membrane so as to regulate the accretion of cholesterol homeostatically (7, 8, 12). However, the mechanisms by which plasma membrane cholesterol controls the ER pool remain to be established. In this study, we tested the hypothesis that PKCs participate in this process.

PKC isoforms, of which there are about a dozen, make a profound contribution to cellular homeostasis (13–17). We therefore tested pharmacologic agents that act upon different PKCs and relevant protein phosphatases (17–19) for their impact on ER cholesterol. The simplest measure of the steady-state level of this pool is the rate of cholesterol esterification *in vivo* (8). We corroborated the results of that analysis by measuring the functional pool of cholesterol in the ER (7, 8). Our evidence suggests that different PKCs exert positive and negative influences on ER cholesterol levels and may therefore play a complex role in cholesterol homeostasis.

MATERIALS AND METHODS

Materials. Human foreskin fibroblasts were cultured as described (5). All PKC and protein phosphatase activators and inhibitors were from Calbiochem except for bryostatin 1 which was obtained from Biomol Research Labs (Plymouth Meeting, PA). [Oleoyl-1-^{14}C]Coenzyme A was purchased from Dupont NEN; [1α,2α (n)-^{3}H]cholesteryl oleate was from Amersham Life Science, Inc. Lipoprotein-deficient serum (LPDS) was prepared from fetal bovine serum (20). Cyclodextrins (CD) were from Research Plus, Inc. (Bayonne, NJ); α-hydroxypropyl-β-CD was used as a vehicle for cholesterol delivery (21).

Assays. For the assay of cholesterol esterification *in vivo,* replicate flasks were preincubated with fresh medium containing 5% LPDS and PKC-directed agents for 2 h (or 1 h for calyculin A). Cell surfaces were then labeled with [^{3}H]cholesterol from an α-hydroxypropyl-β-CD vehicle during a 10-min incubation at room temperature (4). The labeling solution was replaced with growth medium containing 5% LPDS plus agents and the flasks incubated for an additional 1.5 h at 37°C. The fraction of plasma membrane [^{3}H]cholesterol esterified was then deter-

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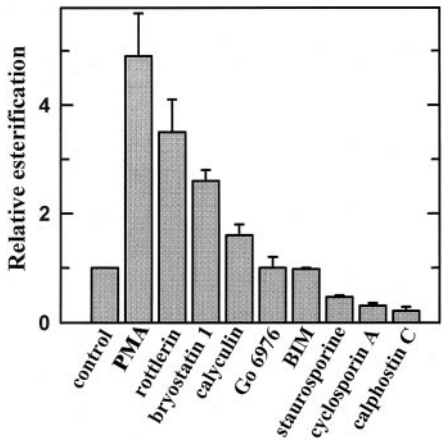

FIG. 1. Effect of PKC agents and phosphatase inhibitors on cholesterol esterification. Bars: 1, control; 2, 0.20 μM PMA; 3, 5 μM rottlerin; 4, 0.20 μM bryostatin 1; 5, 0.003 μM calyculin; 6, 0.030 μM Go 6976; 7, 5,0 μM BIM; 8, 0.030 μM staurosporine; 9, 2.0 μM cyclosporin A; 10, 0.15 μM calphostin C. Values are from three or more experiments.

mined (7); values relative to controls are averages from multiple experiments ± standard deviations.

To vary cell (primarily plasma membrane) cholesterol, replicate flasks were incubated for 10 min at 37°C in medium containing, respectively, 5% LPDS and 0.2% β-methyl-CD or 10% fetal bovine serum, 0.5% BSA and α-hydroxypropyl-β-CD/cholesterol complexes (4). PKC agents were present during this and subsequent incubations. Control flasks were incubated in growth medium plus the solvent blank (DMSO or ethanol at <0.5%). The cholesterol content of the depleted and enriched cells was 70–90 and 130–190% of controls, respectively. The flasks were then incubated for 2 h at 37°C in fresh medium containing the agents and LPDS (depleted cells) or 10% serum (control and enriched cells) as described above.

It takes >1 h for the ER pool to respond to acute changes in cell cholesterol content (8). On the other hand, time-dependent downregulation of PKCs as well as secondary and toxic effects are induced by prolonged exposure to some of these agents. We therefore limited the preincubation and the esterification periods to 2 h or less, even though not all of the PKC agents elicit their full effect during that time. Similarly, high levels of many of the agents caused changes in cell shape and were therefore used at a reduced concentration. PKCs are not known to modify acyl-CoA:cholesterol acyl transferase activity directly at the levels used in Fig. 1 (22, 23), and none of the agents employed affected the activity of the enzyme itself when tested in cell homogenates in the presence of excess cholesterol.

ER cholesterol was determined as described (7, 8) following incubation of fibroblasts for 2–3 h at 37°C in growth medium containing the indicated agents. Values were calculated as the mass of [^{3}H]cholesterol esters per milligram of cell protein and expressed relative to untreated controls ± SD for n experiments. The duplicate assays agreed within 6%.

Protein was determined as described (24).

RESULTS

PKC Agents Affect Cholesterol Esterification

Given the rapid flux of plasma membrane cholesterol through the small pool in the ER, the rate of esterification of [^{3}H]cholesterol pulsed into the cell surface provides a good indication of the time-averaged ER pool size (8, 25). A variety of experimental agents were found to alter the rate of esterification of plasma membrane [^{3}H]cholesterol at concentrations characteristic of their effects on different PKC enzymes (14, 17). PMA and bryostatin 1, activators of conventional and novel PKC isoforms, stimulated cholesterol esterification 4.9- and 2.6-fold, respectively (Fig. 1 and Table 1). Rottlerin was also a positive effector below ~5 μM, in which range it acts selectively on PKC-δ (26), but inhibited at higher concentrations (Fig. 2). Calyculin A, a potent inhibitor of protein phosphatases 1 and 2A (18, 19), also stimulated cholesterol esterification slightly (Fig. 1), possibly through the stabilization of the phosphorylation of activated PKCs or their protein targets.

Inhibitors with selectivity for Ca^{2+}-dependent PKCs (14, 17) did not affect basal cholesterol esterification significantly. These included bisindolylmaleimide I (BIM; also called Gö 6850) and Gö 6976 (Table 1), as well as 0.3 μM Ro 320432 (not shown). In contrast, two

TABLE 1

Effect of PKC Agents, Individually and in Pairs, on Cholesterol Esterification *in Vivo*

Agent (μM)	Relative esterification	SD	n
None	1.0		
PMA (0.20)	4.7	0.4	9
Gö 6976 (0.030)	1.0	0.2	3
BIM (5.0)	0.85	0.15	4
Staurosporine (0.030)	0.54	0.1	7
Cyclosporin A (2.0)	0.52	0.1	5
Calphostin C (0.15)	0.20	0.1	10
PMA (0.20) + Gö 6976 (0.030)	3.6	0.8	3
PMA (0.20) + staurosporine (0.030)	1.7	0.8	3
PMA (0.20) + BIM (5.0)	1.3	0.3	3
PMA (0.20) + calphostin C (0.15)	0.9	0.2	3
Cyclosporin A (2.0) + staurosporine (0.030)	0.26	0.01	3
Calphostin C (0.15) + staurosporine (0.030)	0.14	0.03	3
Cyclosporin A (2.0) + calphostin C (0.15)	0.10	0.02	3

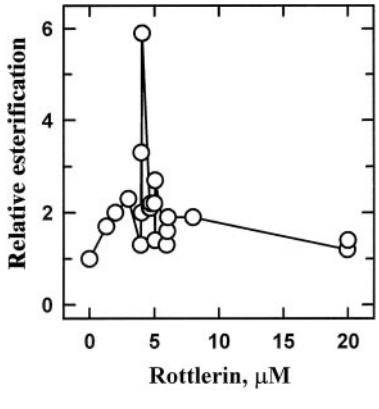

FIG. 2. Effect of rottlerin on cholesterol esterification *in vivo*. Data are individual values from 17 experiments. At 40–80 μM rottlerin, esterification returned to the control value.

TABLE 2

Effect of PKC Agents on *in Vivo* Cholesterol Esterification in Cells with Modified Cholesterol Content

Cell cholesterol	Agent (μM)	Relative esterification[a]	SD
Control	None	4.7	1.0
Depleted	None	1.0	
Depleted	PMA (0.20)	4.6	1.4
Depleted	Bryostatin 1 (0.20)	4.2	1.5
Depleted	Rottlerin (4.0)	2.1	0.2
Control	None	0.16	0.02
Enriched	None	1.0	
Enriched	BIM (5.0)	0.98	0.02
Enriched	Cyclosporin A (2.0)	0.6	0.02
Enriched	Calphostin C (0.15)	0.16	0.10
Enriched	Cyclosporin (2.0) + calphostin C (0.15)	0.07	0.01

[a] Averages ± SD from three experiments.

general PKC inhibitors, staurosporine and calphostin C, blocked cholesterol esterification. Unlike calyculin A (which inhibits protein phosphatases 1 and 2A), cyclosporin A (an inhibitor of protein phosphatase 2B; Ref. 19) inhibited cholesterol esterification (Fig. 1 and Table 1). Gö 6983 reduced cholesterol esterification by one-third at 100 nM (not shown).

To test whether these agents in fact acted upon PKC systems, we examined the action of mixtures of activators and inhibitors. We found that calphostin C and staurosporine opposed the positive effect of PMA on cholesterol esterification (Table 1). Staurosporine also blocked stimulation by bryostatin 1 (not shown). Even though BIM had a minimal effect on basal cholesterol esterification, it prevented stimulation by PMA and bryostatin 1 equally well at ~1 μM (not shown) and 5 μM (Table 1). On the other hand, Gö 6976 had little or no effect on either basal or PMA-stimulated esterification (Table 1).

Certain pairs of inhibitors reduced cholesterol esterification activity more than either agent alone. In the most extreme case, combining cyclosporin A with calphostin reduced cholesterol esterification by 90% (Table 1). These results suggest that there may be little or no cholesterol esterification that is not dependent on PKC; if so, the provision of plasma membrane cholesterol to the ER may depend entirely on PKC-responsive mechanisms.

Combinations of activators had diverse effects on cholesterol esterification. A few pairs (e.g., rottlerin plus bryostatin 1) stimulated somewhat more than either agent alone; other pairs gave values between those of the two agents; some pairs activated less than either agent alone. Treatment with rottlerin plus calyculin A was not stimulatory even though each agent had a positive effect by itself (not shown).

PKC Agents Block Esterification Responses to Variations in Plasma Membrane Cholesterol

Changing cell cholesterol levels with cyclodextrins evokes marked responses in cholesterol esterification due to the homeostatic adjustment of the size of the ER pool (4, 8). We now show that the reduction of cholesterol esterification normally caused by the extraction of plasma membrane cholesterol was prevented by PKC-directed agents that increased basal cholesterol esterification (Table 2). Conversely, agents that decreased cholesterol esterification in resting cells countered, to differing degrees, the rise in esterification seen in cells acutely loaded with exogenous cholesterol. BIM did not block the stimulation of cholesterol esterification by exogenous cholesterol, while cyclosporin A and, especially, calphostin C did so. The addition of PMA or rottlerin, agents that positively affected resting cells (Table 1), did not further stimulate cholesterol esterification in cells loaded with excess exogenous cholesterol (not shown).

Effect of PKC Agents on ER Cholesterol

A variety of agents altered the size of the ER cholesterol pool (Table 3) in the manner predicted from their influence on cholesterol esterification *in vivo* (Fig. 1 and Table 1). That is, activators of cholesterol esterification (PMA, bryostatin 1 and rottlerin) increased ER cholesterol while the inhibitor, calphostin C, decreased it. Consistent with the findings on *in vivo* cholesterol esterification (Table 1), BIM blocked the positive effect of PMA on the ER cholesterol pool but did not alter its basal level (Table 3).

If the positive PKC agents acted simply by stimulating acyl-CoA:cholesterol acyl transferase activity, they would have diminished the pool of ER cholesterol; however, the opposite was observed. The corresponding argument holds for the negatively-acting agents. Therefore, it appears that the alteration in the *in vivo* rates of cholesterol esterification reflected the modulation of ER cholesterol, as expected (22, 23).

TABLE 3

Effect of PKC Agents on ER Cholesterol

Agent (μM)	Relative ER cholesterol	SD	*n*
None	1.0		
PMA (0.20)	2.3	0.3	5
Bryostatin 1 (0.20)	2.3	0.2	3
Rottlerin (5.0)	2.0	0.1	3
BIM (5.0)	1.0	0.1	3
Calphostin C (0.15)	0.45	0.05	5
PMA (0.20) + BIM (5.0)	1.1	0.1	3

TABLE 4

Effect on *in Vivo* Esterification of 25-Hydroxycholesterol and PKC Agents

Agent (μM)	Relative esterification	SD	*n*
None	1.00		
25-Hydroxycholesterol (0.04)	2.1	0.7	4
Cyclosporin A (2.0)	0.44	0.04	3
Calphostin C (0.15)	0.20	0.1	3
25-Hydroxycholesterol (0.04) + cyclosporin A (2.0)	1.25	0.45	4
25-Hydroxycholesterol (0.04) + calphostin C (0.15)	0.55	0.1	3

PKC Agents Counter the Action of Oxysterols

Certain oxysterols stimulate cholesterol esterification and suppress cholesterol accretion, apparently by increasing the level of ER cholesterol (8). For example, 25-hydroxycholesterol caused cholesterol esterification to increase more than two-fold in the experiment shown in Table 4. Calphostin C and cyclosporin A suppressed this stimulation. The presence of rottlerin did not add to the effect of 25-hydroxycholesterol (not shown). Since the action of oxysterols on ER cholesterol could be mediated through an oxysterol binding protein, it is interesting to note that one member of this family appears to be phosphorylated in a staurosporine-sensitive fashion *in vivo* (27).

DISCUSSION

These data suggest that PKCs are instrumental in the regulation of the homeostatic pool of cholesterol in the ER. PKC-directed agents and relevant protein phosphatase inhibitors reduced or increased the rate of cholesterol esterification over a >40-fold range, a greater span than that elicited by extreme variations in cell cholesterol (4, 8). PKC-directed agents caused a parallel variation in ER cholesterol levels of about fivefold. The greater excursion in cholesterol esterification rates than in ER cholesterol levels may be a manifestation of the high-order dependence of acyl-CoA:cholesterol acyl transferase activity on ER cholesterol concentration (22, 23, 28).

Our data suggest that more than one PKC is involved in modulating ER cholesterol. However, they do not permit a definitive identification of the particular isoforms involved. This is because individual agents affect more than one PKC isoform and can elicit complex behavior. Nevertheless, some preliminary inferences can be drawn with regard to the principal PKCs expressed in fibroblasts: alpha and beta1 (in the conventional subclass), delta and epsilon (in the novel subclass), and zeta (in the atypical subclass) (29–32).

The basal level of ER cholesterol seemed to depend on a PKC in that this pool was reduced by the broad-spectrum inhibitors, calphostin C and staurosporine. Basal esterification was not inhibited by agents with a preference for Ca^{2+}-dependent conventional PKC isoforms. The putative Ca^{2+}-independent species is unlikely to be PKC-δ because rottlerin, a selective inhibitor, increased the ER cholesterol pool.

PMA and bryostatin 1 increased ER cholesterol. PMA can act at sites other than PKC (33); however, the reversal of its action by PKC inhibitors makes this unlikely here. The particular PKC activated by PMA and bryostatin 1 is probably not the isoform that raises basal ER cholesterol because stimulation by PMA was reversed by ~1 μM BIM while basal ER cholesterol was unaffected up to 5 μM BIM. Similarly, members of the atypical PKC subclass are not favored because these isoforms are poorly responsive to PMA (14). BIM at ~0.1 μM did not inhibit the action of PMA and bryostatin 1 (not shown); this would seem to rule out a conventional type of PKC, given their extreme sensitivity to this agent. The data therefore suggest a member of the novel subclass of PKCs (14, 17). The isoform in question is unlikely to be delta, however, since rottlerin increased ER cholesterol. PKC-epsilon remains a candidate for this positive effector of ER cholesterol.

The rise in ER cholesterol evoked by excess plasma membrane cholesterol appears to be mediated by a PKC since the stimulation of cholesterol esterification was blocked by calphostin C (Table 2). The isoform in question was not sensitive to BIM; therefore, it presumably was not the same as the isoform that responded to PMA (Table 1).

Could calphostin C act here by inhibiting phospholipase D, thereby inactivating PKC indirectly by blocking the production of diacylglycerol (34)? This is unlikely because the inhibition of phospholipase D would not alter the action of PMA as observed here. It is also unlikely that calphostin C is acting on a protein kinase other than a PKC (34), given its high selectivity (35).

The presence of a negatively-acting PKC was suggested by the positive effect on ER cholesterol of the PKC inhibitor, rottlerin. The delta isoform is its preferred target (26). The reduction of ER cholesterol by the protein phosphatase inhibitor, cyclosporin A, might then reflect the preservation of putative inhibitory phosphorylation at the target site of the PKC-δ. If PKC-δ in fact exerts a negative effect on ER cholesterol, it would presumably not be the mediator of the positive action of PMA, even though the delta isoform is activated by phorbol esters. Indeed, stimulation of the putative negatively-acting PKC-δ by PMA might well have been masked by its greater effect on positively-acting antagonistic kinases. Opposing ef-

fects of delta and epsilon PKCs have been reported in other systems (36).

The negative effect of rottlerin at high concentrations can be ascribed to its weak inhibitory action on isoforms other than delta (37). Rottlerin at 5 μM is also known to inhibit CaM kinase III (38); however, because the only known physiological substrate for this enzyme is elongation factor 2 (39), the effect of rottlerin observed here is likely to be related to PKC-δ. It has also recently been suggested that rottlerin uncouples oxidative phosphorylation, thereby lowering the production of ATP by mitochondria with multiple consequences (40). However, such a mechanism would not explain the biphasic dose–response curve shown in Fig. 2.

How PKCs participate in cholesterol homeostasis remains to be established. There is evidence that the small compartment of cholesterol in the ER is normally swept out rapidly by a brisk throughput from the much larger pool of plasma membrane cholesterol (25). The adjustment of influx and efflux rates could regulate the ambient size of the ER cholesterol pool according to the signaled needs of the plasma membrane (25). A sensor of the cholesterol in the plasma membrane could regulate the activities of PKCs and protein phosphatases so as to adjust the level of phosphorylation of as yet unknown effector proteins acting upon the cholesterol transport pathways between the regulatory and regulated compartments. The PKCs that raise the level of cholesterol in the ER could act either to stimulate influx from the plasma membrane to the ER or slow its exit or both. Negatively acting PKCs would have the opposite effect.

It is reasonable to imagine that the primary signal for homeostatic adjustments arises in the plasma membrane, since that is the principal pool being managed. This idea is appealing, since PKCs are often activated by their association with the lipids in that membrane (13). In that case, plasma membrane phosphoproteins might regulate the inward flux of cholesterol to the ER. However, it is also possible that regulatory sites are associated with shuttle vesicles, with the cytoskeleton or with the ER itself.

One can therefore postulate the following elements in a system for cholesterol homeostasis: (a) A change in the level of cholesterol in the plasma membrane is detected and signaled by a sensor that (b) adjusts the activity of PKCs and/or protein phosphatases so as to (c) tune the level of cholesterol in the ER which, in turn, (d) modulates resident effectors of cholesterol homeostasis, (e) thereby restoring the proper plasma membrane cholesterol level. The sensor, the specific PKC isoforms, the pathway of cholesterol transport, and other upstream elements in this hypothetical system remain to be identified.

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