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ROLE OF VOLTAGE-SENSITIVE CALCIUM CHANNELS IN $[Ca^{2+}]_i$ AND SECRETORY RESPONSES TO ACTIVATORS OF PROTEIN KINASE C IN PITUITARY GONADOTROPHS

Shun-ichiro Izumi, Stanko S. Stojilković, Toshihiko Iida,
Lazar Z. Krsmanović, Robert J. Omeljaniuk, and Kevin J. Catt [1]

Endocrinology and Reproduction Research Branch, National Institutes of Child Health and Human Development, NIH, Bethesda, Maryland 20892

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Summary: The gonadotropin secretory response of anterior pituitary cells to phorbol esters includes both extracellular Ca^{2+}-dependent and -independent components (Stojilković *et al*, 1988; J. Biol. Chem. 263, 17301-17306, 1988). In cultured pituitary cells, measurements of $[Ca^{2+}]_i$ using Fura-2 and of LH release during cell perifusion studies revealed that the initial effects of phorbols and permeant diacylglycerols on these responses are extracellular Ca^{2+}-dependent and are mediated through activation of voltage- and dihydropyridine-sensitive calcium channels. On the other hand, pretreatment with phorbol esters for 30 to 60 min inhibited subsequent $[Ca^{2+}]_i$ responses to diacylglycerols and phorbols and significantly reduced agonist-induced biphasic $[Ca^{2+}]_i$ responses, with no change in the number of GnRH receptors. These findings demonstrate that protein kinase C exerts both positive and negative control of $[Ca^{2+}]_i$, and indicate that the calcium, phospholipid dependent enzyme participates in the activation of voltage-sensitive calcium channels and hormone secretion in pituitary gonadotrophs.

The entry of ionized calcium through selective voltage-sensitive calcium channels (VSCC) is an important mechanism for regulation of the cytosolic calcium concentration ($[Ca^{2+}]_i$) in excitable central and peripheral tissues (1). The passage of calcium may occur through different kinds of VSCC, known as T, N, and L types (2). Although the voltage-sensitivity of these channels is crucial in understanding their kinetics, it has been postulated that VSCC may also be regulated through activation of specific receptors (3). Thus, agonists might activate calcium influx by direct interaction of a receptor with the channels (receptor-coupled) or through a second messenger system. The messenger-activated group includes many examples of VSCC that are modulated by receptor-induced formation of second messengers and/or by activation of protein kinases (4). The possible

[1] *Address correspondence and reprints request to:* Dr Kevin J. Catt, Bldg. 10, Room B1-L400, National Institutes of Health, Bethesda, Maryland 20892.

The abbreviations used are: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; EGTA, [ethylene bis(oxyethylenenitrilo)tetraacetic acid; PMA, phorbol 12-myristate-13-acetate; PDBu, phorbol 12,13-dibutyrate; 4αPDD, 4α-phorbol-12,13, didecanoate; DOG, dioctanoylglycerol; OAG, sn-1-oleoyl-2-acetylglycerol.

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role of phosphorylation in modulation of calcium channel activity has been suggested by studies on cardiac muscle cells (5), in which cAMP is the second messenger involved in mediating beta-adrenergic stimulation of contractility. In general, elevations of cAMP and activation of cAMP-dependent protein kinase cause phosphorylation of VSCC and increased peak calcium current; conversely phosphorylation-dependent activation of VSCC reversed when an endogenous phosphatase is stimulated to dephosphorylate the calcium channels (6).

Electrophysiological studies have confirmed the presence of two membrane potential-sensitive calcium channels in tumoral (7) and normal pituitary cells (8,9), respectively characterized by a fast, transient (T-type) current with rapid activation-inactivation kinetics, and a delayed, dihydropyridine-sensitive (L-type) current. While T channels typically exhibit voltage-dependent activation and inactivation (3,7,9), L-type channels often undergo second messenger-dependent activation and inactivation (3,10). It has been proposed that activation of L-type channels in GH_3 cells is associated with phosphorylation of the calcium channels by cAMP-dependent protein kinase (10). This finding is in agreement with data showing the role of cAMP as one of the second messenger systems in these cells (11), but contrasts with observations in GH_4C_1 tumor pituitary cells that phorbols and diacylglycerols activate the influx of extracellular Ca^{2+} through dihydropyridine-sensitive VSCC (12). In these cells, it was proposed that diacylglycerol might open calcium channels by closure of TRH-regulated potassium channels via activation of protein kinase C.

In pituitary gonadotrophs, agonist-induced LH release by GnRH is partially dependent on extracellular Ca^{2+}, and the maintenance of the secretory response requires calcium entry through dihydropyridine-sensitive Ca^{2+} channels (13). LH release and calcium entry are also stimulated by tumor-promoting phorbol esters (14). Since GnRH receptors are not directly coupled to the cAMP-dependent pathway (15) but to stimulation of phospholipase C and production of $InsP_3$ (16) and diacylglycerol (17), it is possible that activation of protein kinase C promotes calcium influx through L-type channels in pituitary gonadotrophs (18). To test this hypothesis, we analyzed the effects of phorbol esters and permeant diacylglycerols on calcium entry and gonadotropin release, as well as on the component of LH secretion that is independent of a concominant increase in $[Ca^{2+}]_i$.

EXPERIMENTAL PROCEDURES

Anterior pituitary glands obtained from adult female rats 2 weeks after ovariectomy were used for the preparation of primary cultures of pituitary cells. Groups of 50 glands were minced and subjected to enzymatic dispersion with trypsin (Sigma, St. Louis, MO; 16); cells were suspended in medium 199 (M199, Gibco, Grand Island, NY) containing 10% horse serum, penicillin and streptomycin, and cultured at 37 °C under 5% CO_2-95% air. Cell viability averaged 90% as assessed by trypan blue staining. For perifusion studies, 10 x 10^6 cells were incubated with preswollen Cytodex-1 beads (Pharmacia LKB Biotech. Inc., Piscataway, NJ) for 3 days. The beads were then collected and resuspended in Hanks'-M199 with HEPES (25 mM) and 0.1% BSA (Miles Lab, Elkhart, IN), and loaded into a 500 µl perifusion chamber (Endotronics, Minneapolis, MN). Cells were perifused with the same medium for 120 min at a flow rate of 0.6 ml/min, and then with the

appropriate medium for each experiments. Phorbol esters were dissolved in dimethyl sulfoxide and added to the perifused cells in 1 µl aliquots to a total incubation volume of 10 ml . Each set of experiments was performed with the same batch of cells. For analysis of the effects of extracellular calcium, three different media were employed; Ca^{2+}-free medium (prepared without addition of calcium salts by Media and Tissue Unit, NIH; total Ca^{2+}=5 µM determined by calcium electrode); Ca^{2+}-free medium with addition of 1.25 mM calcium salts (employed as standard medium), and Ca^{2+}-free medium containing EGTA (calculated free Ca^{2+} was 200 nM, according to the Kd of EGTA and pH of the medium). Fractions were collected every 15 seconds or 1 min and stored at -20 °C prior to LH assay, using the reagent and standards provided by the National Pituitary Agency, Baltimore, MD. Hormone release was expressed as ng/ml.

Measurements of $[Ca^{2+}]_i$ were performed in dispersed pituitary cells from ovariectomized rats, which contain about 25% gonadotrophs. Ten million cells were resuspended in 10 ml M199 containing 25 mM HEPES and 0.3 mM BSA and incubated with 1 µM Fura 2-AM for 30 min at 37 °C. They were then diluted and centrifuged at 200 x g, washed twice in assay buffer (Hanks' salts with 25 mM HEPES and 0.01% BSA) and kept on ice before use. 1-2 million cells were used for $[Ca^{2+}]_i$ assay by fluorescence analysis (dual excitation wavelenght; 340 and 390 nm) in a 3 ml cuvette in an SLM 8000C spectrofluorometer (SLM Instruments, Inc., Urbana, IL) fitted with a magnetic stirrer and thermostatted cuvette holder. $[Ca^{2+}]_i$ values were calculated after correction of emission data collected at 500 nm for dye leakage and autofluorescence, according to formulae described by Grynkiewich et al (19).

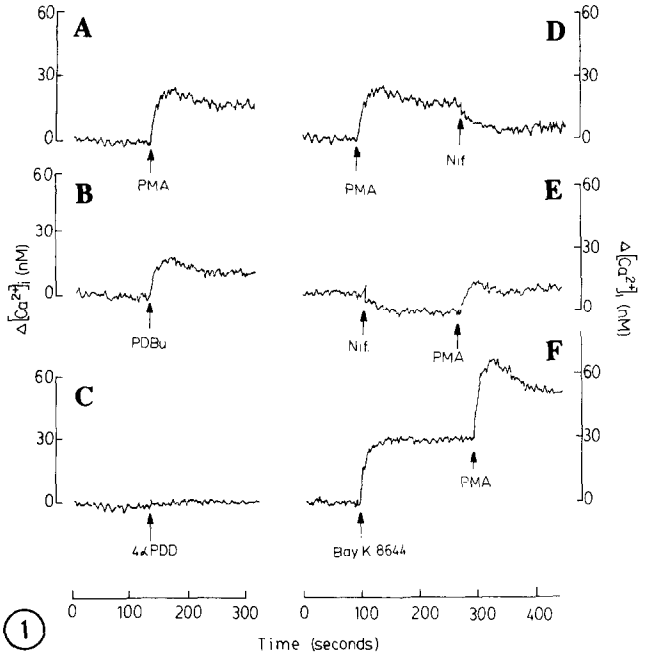
Freshly prepared rat pituitary cells were washed twice with prewarmed Hanks'-M199 and incubated in the same type of medium with vehicle (dimethyl sulfoxide, 0.01% final) or a given concentration of PMA for various times at 37 °C. At the end of the incubation, 40 ml of ice-cold Hanks' M199 was added to the cells, which were then centrifuged at 400 g x 10 min and washed again with ice-cold Hanks'-M199. The cell pellet was resuspended with GnRH receptor assay buffer (Tris, 10 mM; DTT, 1 mM; BSA 0.1%, pH=7.6), then sonicated and dispensed into 1.5 ml centrifuge tubes. The broken cell suspension was incubated with ^{125}I-GnRH agonist in the presence or absence of 100 nM [D-Ala^6]desGly^{10}-GnRH-NEt (for non-specific binding) for 2 h on ice (20). The binding reaction was terminated by addition of 1 ml of ice-cold receptor assay buffer, followed by centrifugation at 13,000 g x 10 min at 4 °C; the supernatants were aspirated and the bottoms of the tubes containing the cell pellet were clipped off and bound radioactivity was measured by gamma-spectroscopy.

RESULTS

Effects of phorbol esters and permeant diacylglycerols on $[Ca^{2+}]_i$

We have previously described the ability of the protein kinase C activator, PMA, to increase $[Ca^{2+}]$i in anterior pituitary cells (14). This effect of PMA was dependent on extracellular calcium, indicating the role of protein kinase C in promotion of Ca^{2+} influx. As shown in Fig. 1A, PMA (1 µM) induced a rapid increase in $[Ca^{2+}]_i$ within 30 s. Another active phorbol ester, PDBu, exibited similar effects (Fig. 1B). On the other hand, 4aPDD did not alter $[Ca^{2+}]_i$ at concentrations up to 100 nM (Fig. 1C). DOG (1 µM, Fig. 2A) and OAG (1 µM, Fig. 2B) elicited initial increases in $[Ca^{2+}]_i$ similar to those induced by phorbol esters. The elevations of $[Ca^{2+}]_i$ by DOG and OAG were prevented by lowering $[Ca^{2+}]_e$ to 200 nM with EGTA before addition of the permeant diacylglycerols (not shown).

Both phorbol ester- and diacylglycerol-induced increases in $[Ca^{2+}]_i$ were found to be dihydropyridine-sensitive, suggesting the participation of L-type calcium channels in the action of protein kinase C activators. After treatment with PMA (Fig. 1D), DOG (Fig. 2A)



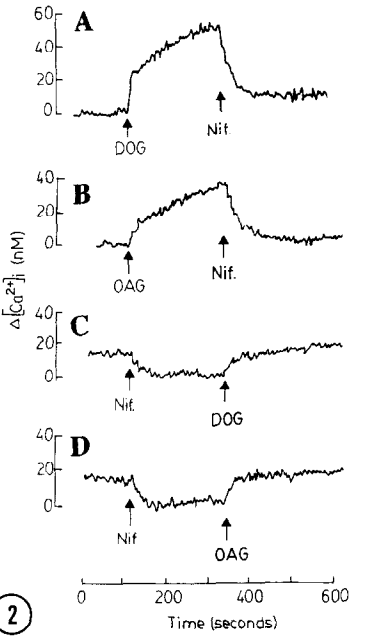
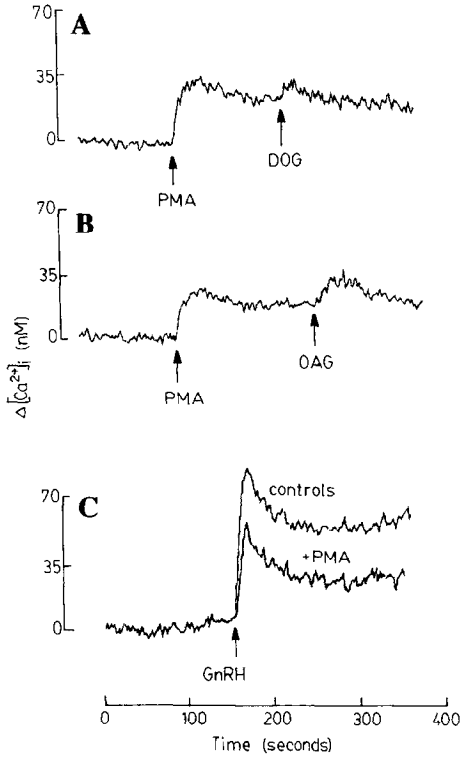


Fig. 1. *Acute effects of phorbol esters and dihydropyridines on $[Ca^{2+}]_i$. A,B,C; phorbol ester-induced increases in intracellular calcium. Phorbols were added in final concentrations of 1 μM. D,E,F; modulation of the action of PMA on $[Ca^{2+}]_i$ by calcium channel antagonist (nifedipine, 1 μM, Nif) and agonist (Bay K 8644, 1 μM) analogs. The mean resting level of $[Ca^{2+}]_i$ was 102 ± 7 nM. Traces are representative of experiments performed with similar results in three different batches of pituitary cells.*

Fig. 2. *Blockade of the actions of permeant diacylglycerols, DOG and OAG (1μM), on $[Ca^{2+}]_i$ by nifedipine (1 μM) in pituitary cells. Basal $[Ca^{2+}]_i$ was 105 ± 7 nM. The results shown are representative of three similar experiments.*

or OAG (Fig. 2B) the calcium channel antagonist, nifedipine (1 μM), reduced the elevated $[Ca^{2+}]_i$ almost to the basal level. Nifedipine alone decreased basal $[Ca^{2+}]_i$ by about 30%, due to inhibition of basal influx of extracellular Ca^{2+}. In nifedipine-pretreated cells, the effects of PMA, DOG and OAG on $[Ca^{2+}]_i$ were reduced to less than 20% of the elevations observed when these agents were added alone (Fig. 1E and 2C,D). Thus, the $[Ca^{2+}]_i$ responses to phorbols and permeant diacylglycerols are predominantly due to influx of extracellular Ca^{2+} through dihydropyridine-sensitive calcium channels. The presence of additivity between the actions of PMA and the calcium channel agonist, Bay K 8644, further supports this conclusion (Fig. 1F). However, the participation of another, minor, Ca^{2+} entry pathway dependent on protein kinase C action cannot be excluded.

After PMA treatment for 2-3 min, the $[Ca^{2+}]_i$ responses to DOG and OAG were markedly attenuated (Fig. 3 A,B). In another experiment, the cells were pretreated with PMA (1 μM) for 30 min, then washed and loaded with Fura-2 AM. In such PMA-pretreated cells, increases of $[Ca^{2+}]_i$ induced by PMA or DOG were completely abolished (not shown) and the GnRH-induced biphasic $[Ca^{2+}]_i$ response was significantly reduced (Fig. 3C). In a parallel experiment, the number of GnRH receptors (estimated by specific



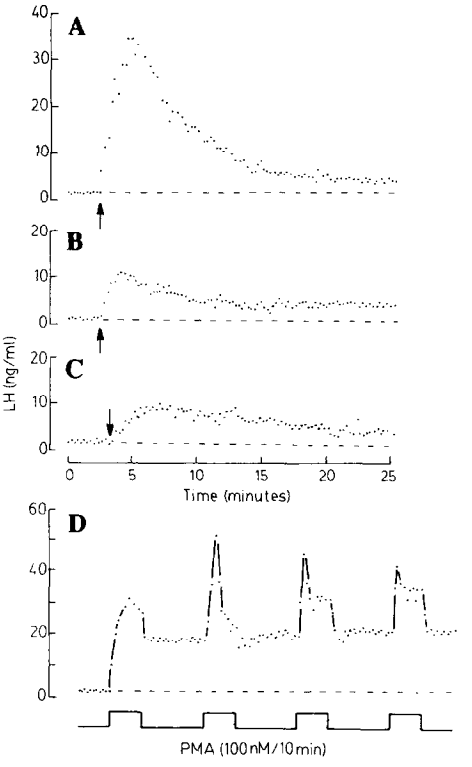


Fig. 3. Effects of pretreatment with PMA on $[Ca^{2+}]_i$ responses to diacylglycerols and phorbols. A,B; $[Ca^{2+}]_i$ was measured after addition of PMA (1 μM) and subsequent stimulation with DOG and OAG (1 μM). C; Pituitary cells were exposed to PMA (1 μM) for 30 min, washed and loaded with Fura-2 for 30 min, and then stimulated with GnRH (100 nM). Similar changes were observed in 3 independent experiments.

Fig. 4. Effects of PMA on LH release. A; Control, LH release induced by 1 μM PMA in the presence of 1.25 mM extracellular calcium. B,C; Abolition of extracellular calcium-dependent action of PMA on gonadotropin release in low-calcium (200 nM) medium (panel B) or in the presence of 1 μM nifedipine (panel C). Ca^{2+}-deficient medium or nifedipine were applied 5 min before PMA stimulation. Arows indicate the beginning of stimulation with PMA. D; Repetitive stimulation of pituitary cells with 100 nM PMA for 10 min, with 20 min washing periods between stimuli. Fractions were collected every 15 sec in all experiments. The results shown are representative of three similar experiments.

binding of the ^{125}I-GnRH analog to cultured pituitary cells) was not significantly affected by PMA treatment (controls 100 ± 7.2% vs. treated 96.6 ± 5%; means ± S.E. of data from 4 independent experiments, each done in triplicate). These findings indicate that the ability of protein kinase C activators to elevate $[Ca^{2+}]_i$ is greately reduced when the enzyme has been previously activated by high doses of PMA. The inability of the physiological agonist to elicit the normal biphasic $[Ca^{2+}]_i$ response in such cells further indicates the possible participation of the diacylglycerol-protein kinase C pathway in GnRH-induced calcium entry.

Kinetics of phorbol ester-stimulated gonadotropin release

We have previously reported that phorbol ester-stimulated gonadotropin release from cultured pituitary cells has both extracellular Ca^{2+}-dependent and -independent

components (14). In the present study, the kinetics and calcium-dependence of PMA-induced LH release were analyzed in perifused pituitary cells. In the presence of extracellular Ca^{2+}, application of PMA (1 μM, 10 min) caused a rapid increase in LH release to the maximum value within 60 s, followed by a gradual decrease in the rate of secretion to the steady-state level (Fig. 4A). This acute effect of PMA was predominantly dependent on extracellular Ca^{2+}; the secretory responses of the perifused cells were attenuated by lowering $[Ca^{2+}]_e$ to 200 nM with EGTA (Fig. 4B), or by blocking VSCC with nifedipine (Fig. 4C). Either calcium-free medium or nifedipine was applied 5 min before and during the 10 min pulse of 1 μM PMA.

LH secretory responses to repetitive pulses of PMA (100 nM, 10 min, 2 h^{-1}) are shown in Fig. 4D. At this dose, PMA induced a series of LH responses, but with decreasing peak amplitudes. After removing the secretagogue, LH release was reduced but did not return to the basal level. At higher concentrations of PMA (1 μM), progressive attenuation of the secretory profiles was observed during repetitive stimulation, as well as an increase in basal LH release between the pulses. This increase in basal release was predominantly independent of extracellular Ca^{2+} (not shown), indicating that once activated by saturating doses of PMA, protein kinase C participates in the process of exocytosis not only through modulation of Ca^{2+} entry and calcium-dependent mechanisms of hormone release but also through other steps in exocytosis that do not necessarily require increases in $[Ca^{2+}]_i$.

DISCUSSION

In pituitary gonadotrophs, application of increasing depolarizing voltage pulses during whole-cell patch clamp recording evokes an inward current that is carried by calcium. Turn-off of the current in these cells is typically expressed as a calcium tail current following repolarization (9). The complexity of the temporal course of the inward calcium current is attributable to the combined effects of influx through more than one pathway; further analysis of the voltage-sensitive calcium current revealed that it includes a fast, transient, T component, and a delayed, long-lasting, L component. In gonadotrophs, as in other pituitary cells (7-9), N-type calcium channels are not detectable.

L-type channels are dihydropyridine-sensitive (2,3) and participate in both high K^+- and agonist-induced entry of extracellular Ca^{2+} (13). Since GnRH does not initiate Ca^{2+}-dependent electrical activity, further increases in Ca^{2+} influx during agonist stimulation cannot be explained by increased frequency of action potentials (21). We have proposed that activation of GnRH-receptors increases calcium influx by modulating the activity of dihydropyridine-sensitive calcium channels (22). The present data are in agreement with this proposal, and show that active phorbol esters and permeant diacylglycerols also promote the entry of calcium through dihydropyridine-sensitive calcium channels. Since GnRH receptors are coupled to polyphosphoinositide hydrolysis and production of the second messengers, $InsP_3$ and diacylglycerol (16,17), it is possible that increased levels of diacylglycerol and cytosolic Ca^{2+} activate protein kinase C, which in turn phosphorylates

dihydropyridine-sensitive calcium channels and promotes the entry of Ca^{2+}. Such a mechanism has also been suggested by studies on the actions of GnRH and PMA in single gonadotrophs, in which the second phase of the $[Ca^{2+}]_i$ response to GnRH was enhanced by the phorbol ester in a nifedipine-sensitive manner (18).

The present data clearly show that the stimulatory actions of phorbols and permeant diacylglycerols are transient, and that prolonged or repetitive stimulation with PMA is followed by attenuation of the $[Ca^{2+}]_i$ signal and the gonadotropin response. Pretreatment with phorbols also reduced the actions of GnRH, as well as OAG and DOG, on calcium entry. Since the number of GnRH receptors was not significantly changed by treatment with PMA, it is possible that such impairment of the ability of GnRH and the kinase C activators to increase $[Ca^{2+}]_i$ results from inactivation of Ca^{2+} channels by the sustained action of the calcium, phospholipid-dependent enzyme. Recently, phosphorylation by protein kinase C has been postulated to regulate both the activation and inactivation properties of dihydropyridine-sensitive calcium channels in other systems. For example, neonatal rat ventricular myocytes respond to PMA with an increase in the probability of opening for single Ca^{2+} channel currents, probably as the result of phosphorylation of dihydropyridine-sensitive calcium channels (23). In contrast, PMA inhibits calcium current in PC 12 cells (24) and chicken sensory neurons (25), suggesting that phosphorylation can also cause Ca^{2+} channel inactivation. However, such inhibitory effects of PMA could also result from down-regulation of protein kinase C (26), which has been proposed as the explanation for the sustained inhibitory action of phorbol esters on calcium current in neonatal rat ventricular myocytes (23).

After addition of saturating doses of nifedipine, phorbol esters are still able to provoke an attenuated influx of Ca^{2+}. This minor (15-20% of control) dihydropyridine-insensitive Ca^{2+} entry pathway(s) could be attributable to the presence of other types of Ca^{2+} channels (T or receptor-operated), as previously discussed (1). Alternatively, such Ca^{2+} entry pathways could be associated with protein kinase C-mediated modulation of Na^+-Ca^{2+} or H^+-Ca^{2+} exchangers, which are known to be present in some populations of pituitary cells (27).

LH secretory profiles, measured in phorbol ester-stimulated cells show a general correlation with $[Ca^{2+}]_i$, consistent with the proposed role of this ion in the regulation of LH exocytosis. The majority of the PMA and PDBu-induced LH release is extracellular Ca^{2+}-dependent and nifedipine-sensitive. However, addition of nifedipine or omission of Ca^{2+} from the extracellular medium attenuates, but does not abolish, the secretory response to phorbol esters. The contribution of this extracellular Ca^{2+}-independent component of phorbol ester action to LH exocytosis represents no more than 10-20% during the first 5 minutes of stimulation. On the other hand, after 3 hr stimulation of static cultures by 1 μM PMA (14), the extracellular Ca^{2+}-independent and -dependent components of LH release were 70% and 30%, respectively (14) This difference between the acute and long-term effects of PMA is caused by two factors, the increased extracellular Ca^{2+}-independent component of LH release during prolonged or frequent stimulation with phorbols, and the

decreased extracellular-Ca^{2+}-dependent and nifedipine-sensitive component due to inactivation of L-type calcium channels. The development of channel inactivation is a relatively rapid process (occurring within 10-15 min) and it is not surprising that under prolonged static culture conditions, nifedipine has little effect on PMA-induced LH release (14). At low doses, phorbol ester-induced LH release is rapidly reversible. In contrast, the secretory mechanism remains active for longer periods in cells exposed to high doses of PMA, and the major part of this secretion is extracellular Ca^{2+}-independent.

In summary, phorbol esters and permeant diacylglycerols induce a complex pattern of $[Ca^{2+}]_i$ and LH release in primary cultures of pituitary gonadotrophs. The actions of these activators of protein kinase C are include both positive and negative modulation of dihydropyridine-sensitive calcium channels, and also the activation of another Ca^{2+} entry pathway, as well as steps that do not require Ca^{2+} influx into the cytosol. We propose that modulation of VSCC by activation of protein kinase C participates in agonist-mediated exocytosis and also in the rapid desensitization that is a prominent feature of pituitary gonadotrophs exposed to high and/or constant levels of GnRH agonists (28). Further studies will be required to characterize the extracellular Ca^{2+}-independent action of protein kinase C, which could be an important factor in the amplification of the $[Ca^{2+}]_i$ signal that occurs in agonist-stimulated cells (29). The ability of phorbol esters to promote a minor degree of calcium entry in the presence of nifedipine indicates the need for further investigation of other Ca^{2+} entry pathway(s) and their significance during agonist stimulation of gonadotropin release.

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