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Calcium stimulates luteinizing-hormone (lutropin) exocytosis by a mechanism independent of protein kinase C

P. Anton VAN DER MERWE,* Robert P. MILLAR and James S. DAVIDSON
Medical Research Council Regulatory Peptides Research Unit, Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, Cape Town, South Africa

Using permeabilized gonadotropes, we examined whether Ca^{2+}-stimulated luteinizing-hormone (LH) exocytosis is mediated by the Ca^{2+}-activated phospholipid-dependent protein kinase (protein kinase C). In the presence of high $[Ca^{2+}]_{free}$ (pCa 5), α-toxin-permeabilized sheep gonadotropes secrete a burst of LH and then become refractory to maintained high $[Ca^{2+}]_{free}$. The protein kinase C activator phorbol myristate acetate (PMA) is able to stimulate further LH release from cells made refractory to high $[Ca^{2+}]_{free}$, suggesting that Ca^{2+} does not stimulate LH release by activating protein kinase C. Staurosporine, a protein kinase C inhibitor, inhibited PMA-stimulated (50% inhibition at 20 nM), but not Ca^{2+}-stimulated, LH exocytosis. In cells desensitized to PMA by prolonged exposure to a high PMA concentration, Ca^{2+}-stimulated LH exocytosis (when corrected for depletion of total cellular LH) was not inhibited. Ba^{2+} was able to stimulate LH exocytosis to a maximal extent similar to Ca^{2+}, although higher Ba^{2+} concentrations were necessary. Ba^{2+} and Ca^{2+} stimulated LH exocytosis with a similar time course, and both were inhibitory at high concentrations. Furthermore, cells made refractory to Ca^{2+} were also refractory to Ba^{2+}. These data strongly suggest that Ba^{2+} and Ca^{2+} act through the same mechanism. Since Ba^{2+} is a poor activator of protein kinase C, these findings are additional evidence against a major role for protein kinase C in mediating Ca^{2+}-stimulated LH exocytosis.

INTRODUCTION

Ca^{2+} is the dominant regulator of secretory exocytosis in a variety of cells (for reviews, see Berridge, 1985; Baker & Knight, 1986). The binding of GnRH to specific receptors on the gonadotrope plasma membrane stimulates a rise in cytosolic $[Ca^{2+}]_{free}$ (Limor *et al.*, 1987; Shangold *et al.*, 1988; Tasaka *et al.*, 1988) which is thought to be the major stimulus for LH exocytosis (for review, see Huckle & Conn, 1988). The mechanism by which Ca^{2+} stimulates exocytosis is not known, but one possibility is via activation of the Ca^{2+}-sensitive protein kinase, protein kinase C (Baker & Knight, 1986; Nishizuka, 1986). In view of the absence of specific inhibitors of protein kinase C (Rando, 1988), studies on a putative role for protein kinase C in Ca^{2+}-stimulated exocytosis have relied on the down-regulation of protein kinase C by prolonged incubation with phorbol esters (Phillips & Jaken, 1983; Rodriguez-Pena & Rozengurt, 1984). Ca^{2+}-stimulated exocytosis was decreased after protein kinase C down-regulation in adrenal chromaffin cells (Burgoyne *et al.*, 1988), but not in PC12 cells (Matthies *et al.*, 1988) nor in pancreatic islets (Hii *et al.*, 1987), suggesting heterogeneity in the mechanism of Ca^{2+}-stimulated exocytosis. However, experiments utilizing protein kinase C down-regulated cells have several drawbacks (see the Discussion section), which makes their interpretation difficult. This is illustrated by the conflicting results obtained in two studies which used protein kinase C down-regulated anterior pituitary cells to investigate the requirement for protein kinase C in GnRH-stimulated LH exocytosis (McArdle *et al.*, 1987; Stojilkovic *et al.*, 1988).

We have previously characterized Ca^{2+}- and phorbol-ester-stimulated LH exocytosis in sheep anterior pituitary cells permeabilized with *Staphylococcus aureus* α-toxin (van der Merwe *et al.*, 1989). In the present study we have used several different approaches, in addition to protein kinase C down-regulation, to provide evidence that Ca^{2+} stimulates LH exocytosis by a mechanism independent of protein kinase C activation.

EXPERIMENTAL

Materials

Purified freeze-dried *Staph. aureus* α-toxin was obtained from Dr. Sucharit Bhakdi (Institute of Medical Microbiology, Justus-Liebig University, Giessen, Germany) and was stored at −20 °C. Staurosporine, obtained from Boehringer-Mannheim (Mannheim, Germany), was dissolved in Me_2SO at 1 mM and stored at 4 °C. Sheep LH (NIADDK-oLH-1-3) and antiserum against it (NIADDK-anti-oLH-1) were kindly provided by the NIDDK (through the National Hormone and Pituitary Program, University of Maryland School of Medicine). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A).

Methods

Cell culture. Primary sheep anterior pituitary cell cultures were prepared as described previously (van der Merwe *et al.*, 1989). Briefly, fresh pituitaries were dispersed by collagenase digestion, plated at a density of 4×10^5 cells/well in 12-well cell-culture plates (Nunc, Copenhagen, Denmark) and used after 48 h of culture in minimal essential medium containing 10% (v/v) fetal-calf serum (Gibco), penicillin (60 mg/l), and streptomycin (100 mg/l), under CO_2/air (1:19).

Permeabilization and cell stimulation. Anterior pituitary cells were permeabilized and stimulated as described by van der Merwe *et al.* (1989). Briefly, the cells were washed twice with Buffer I and then once in Ca^{2+}-free Buffer I. Buffer I comprised (mM): NaCl, 140; KCl, 4; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 8.3; Hepes, 20 (pH 7.4); Phenol Red, 6 mg/l; and 0.1% (w/v) BSA. The cells were then permeabilized by incubation for 10 min

Abbreviations used: GnRH, gonadotropin-releasing hormone (gonadoliberin); LH, luteinizing hormone (lutropin); PMA, phorbol 12-myristate 13-acetate; Me_2SO, dimethyl sulphoxide.

* To whom correspondence should be addressed.

at 37 °C in intracellular (IC) buffer containing 3 μg of α-toxin/ml, 0.5 mM-EGTA, 6.5 mM-$MgCl_2$ and 6 mM-Na_2ATP. IC buffer comprised (mM): sodium propionate, 140; KCl, 4; Pipes (sodium salt), 25 (pH 6.6); Phenol Red, 6 mg/l; and 0.1% BSA. In experiments using staurosporine (Figs. 2 and 3) cells were permeabilized for 20 min. The cell-culture plates were cooled on ice for 10 min and then equilibrated with ice-cold stimulation buffer for 30 min. Stimulation buffer comprised IC buffer with $MgCl_2$ (6.5 mM), Na_2ATP (6 mM) and 30 mM-CaEGTA or -BaEGTA buffers with the indicated $[Ca^{2+}]_{free}$ and $[Ba^{2+}]_{free}$. LH exocytosis was initiated by replacing the stimulation buffer with identical buffer at 37 °C. After the indicated time (usually 10 min) the medium was removed, detached cells were pelleted (400 *g*, 5 min), and the supernatant was stored at −20 °C until LH determination by radioimmunoassay (van der Merwe *et al.*, 1989). Total cellular LH was measured after solubilizing the cells in Nonidet P40 (1%, v/v). LH released is expressed throughout as a percentage of the total cellular LH present at the beginning of the stimulation period.

CaEGTA and BaEGTA buffers. The indicated $[Ca^{2+}]_{free}$ values were obtained by using CaEGTA buffers with various Ca^{2+}/EGTA ratios. The required ratios were calculated by using a computer program written by us, employing metal-ion stability constants for ATP and EGTA (Martell & Smith, 1976; Fabiato, 1981) which were adjusted for use at 37 °C by using enthalpy changes, where available, as described by Martell & Smith (1976). CaEGTA buffers of various Ca^{2+}/EGTA ratios were prepared by mixing stocks of Ca-free EGTA and CaEGTA(1:1) (Ca^{2+}/EGTA ratio 1:1) prepared from the same batch of EGTA and which therefore had identical EGTA concentrations (160 mM, assuming 97% EGTA purity). CaEGTA(1:1) was prepared by end-point titration with calcium oxalate precipitation as the end-point (Miller & Smith, 1984). BaEGTA buffers were prepared in the same way, except that BaEGTA(1:1) was prepared by mixing weighed quantities of $BaCl_2$ and EGTA.

Data presentation. All data shown are representative results from experiments performed three to five times. Data points and error bars represent the mean and range of duplicate determinations. The absence of error bars indicates that the range was smaller than the dimensions of the symbol.

RESULTS

When permeabilized gonadotropes which have been equilibrated at 0 °C with high $[Ca^{2+}]_{free}$ (pCa 5) are warmed to 37 °C, there is burst of LH exocytosis (Fig. 1). LH release is maximal during the first 5 min and declines to basal levels after 20 min, despite the continued presence of high $[Ca^{2+}]_{free}$. This refractoriness to Ca^{2+} could be due either to a depletion of Ca^{2+}-sensitive LH stores or to desensitization of the exocytotic apparatus to Ca^{2+}. Addition of the protein kinase C-activating phorbol ester PMA to cells made refractory to Ca^{2+} resulted in further release of LH. The quantity of LH released from refractory cells in response to PMA was comparable with that released from cells kept at low $[Ca^{2+}]_{free}$ (pCa 8) (Fig. 1), which suggests that the PMA-sensitive LH stores are not markedly depleted by prolonged exposure to high $[Ca^{2+}]_{free}$.

This finding demonstrates that Ca^{2+} and PMA stimulate LH exocytosis, at least in part, by different mechanisms. Since PMA presumably acts by activating protein kinase C, this suggests that Ca^{2+} acts independently of protein kinase C activation. In agreement with this finding, staurosporine, a potent, though non-specific, inhibitor of protein kinase C (Tamaoki *et al.*, 1986), blocked PMA-stimulated LH exocytosis (50% inhibition at

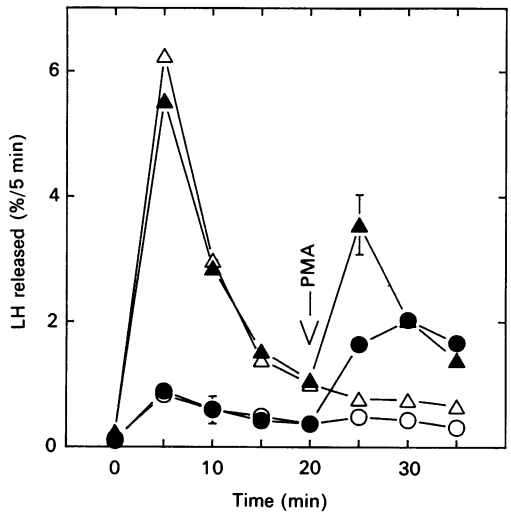

Fig. 1. PMA-stimulated LH exocytosis in cells refractory to Ca^{2+}

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with pCa 8 (○, ●) or 5 (△, ▲). LH exocytosis was initiated by replacing with identical buffer at 37 °C, which was exchanged at 5 min intervals. Buffer added from $t = 20$ min onwards included PMA (100 nM) (●, ▲) or vehicle (0.05% Me_2SO) (○, △). The values at each time point represent the LH released during the preceding 5 min interval. The $t = 0$ point represents the rate of LH release (per 5 min) during the cold equilibration period.

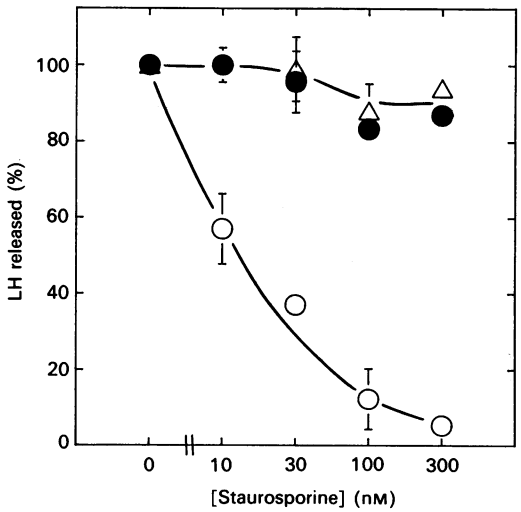

Fig. 2. Effect of staurosporine on PMA-, Ca^{2+}- and Ba^{2+}-stimulated LH exocytosis

Staurosporine was added at the indicated concentration 5 min after the start of the permeabilization step and was present until the end of the experiment. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with pCa 8 plus 100 nM-PMA (○), pCa 5 (■), or pBa 3 (△). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and LH released over the next 10 min was measured. LH release is expressed as a percentage of the release in the absence of staurosporine. Control values were (% of cellular LH, ±range): pCa 5, 15.7±0.3; 100 nM-PMA (pCa 8), 4.3±0.3; pBa 3, 10.2±0.2.

20 nM), but had little effect on Ca^{2+}-stimulated LH exocytosis (Fig. 2). Staurosporine also blocked the enhancement by PMA of Ca^{2+}-stimulated exocytosis over a range of Ca^{2+} concentrations, while having little effect on Ca^{2+}-stimulated exocytosis itself (Fig. 3).

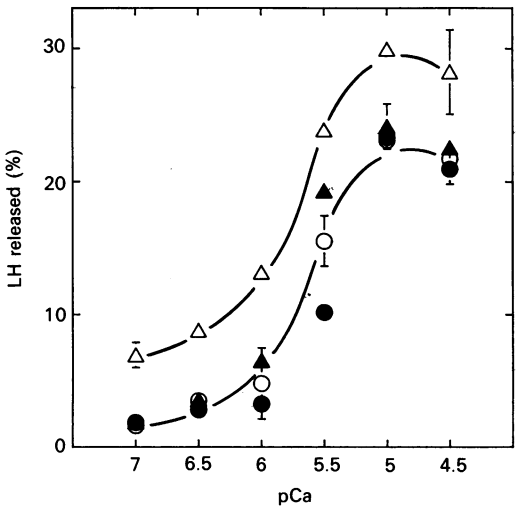

Fig. 3. Effect of staurosporine on combined Ca^{2+}- and PMA-stimulated LH exocytosis

Staurosporine (1 μM) (■, ▲) or vehicle (0.1% Me_2SO) (○, △) was added 5 min after the start of the permeabilization step, and was present until the end of the experiment. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing the indicated $[Ca^{2+}]_{free}$ and 100 nM-PMA (△, ▲) or vehicle (0.05% Me_2SO) (○, ●). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and LH released over the next 10 min was measured.

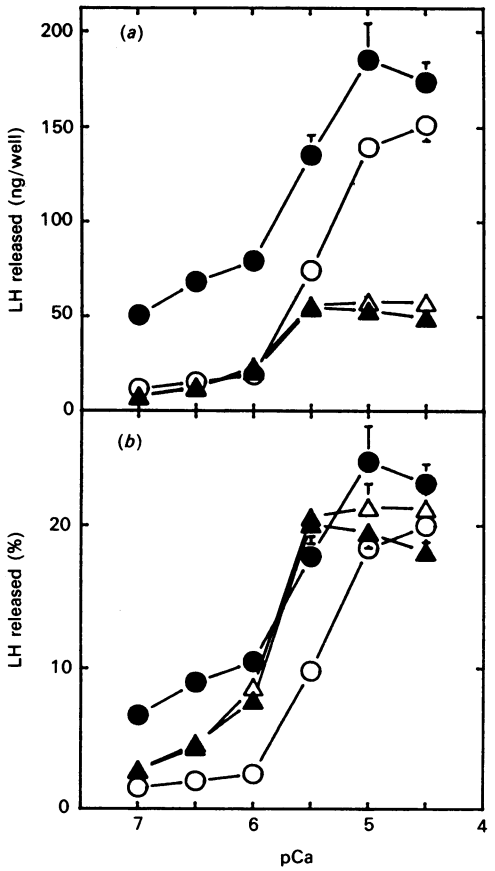

Fig. 4. Effect of PMA desensitization on Ca^{2+}-stimulated LH exocytosis

Pituitary cells were exposed to PMA (500 nM) (△, ▲) or vehicle (0.25% Me_2SO) (○, ●) for 24 h, after which the cells were washed and permeabilized. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with the indicated $[Ca^{2+}]_{free}$ and 100 nM-PMA (●, ▲) or vehicle (0.05% Me_2SO) (○, △). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and LH released over the next 10 min was measured. In (*a*) absolute amounts of LH released are shown, whereas in (*b*) LH release is expressed as a percentage of cellular LH present immediately before stimulation.

Cells incubated for prolonged periods with high concentrations of PMA become desensitized to PMA as a result of proteolytic degradation of cellular protein kinase C (Phillips & Jaken, 1983; Rodriguez-Pena & Rozengurt, 1984; Melloni *et al.*, 1986; Young *et al.*, 1987). Anterior pituitary cells exposed to a high concentration of PMA for 24 h did not release LH in response to subsequent stimulation with PMA, indicating desensitization (Fig. 4*a*). Ca^{2+} was still able to stimulate LH exocytosis in these PMA-desensitized cells, although the absolute amount of LH released was decreased (Fig. 4*a*). This decrease may reflect, in part, a depletion of releasable LH, since cells desensitized to PMA contained less cellular LH [271 ± 15 ($n = 4$) versus control 758 ± 13 ($n = 16$) ng/well (mean ± S.E.M.)]. When LH release was normalized as a percentage of cellular LH present immediately before stimulation, Ca^{2+}-stimulated exocytosis was not inhibited in PMA-desensitized cells (Fig. 4*b*).

Since Ba^{2+} is a poor activator of protein kinase C *in vitro* (Takai *et al.*, 1979; Sekiguchi *et al.*, 1988), and yet is a powerful LH secretagogue in intact pituitary cells (Davidson *et al.*, 1987*b*; Smith *et al.*, 1989), we examined Ba^{2+}-stimulated LH exocytosis in permeabilized cells to determine whether Ba^{2+} activates the same exocytotic mechanism as Ca^{2+} does. High $[Ba^{2+}]_{free}$ stimulated LH exocytosis in permeabilized gonadotropes with a time course very similar to that of Ca^{2+}-stimulated LH exocytosis (Fig. 5*a*). As was the case with Ca^{2+} (Fig. 1), cells eventually became refractory to high $[Ba^{2+}]_{free}$ (Fig. 5*a*), but could still release LH in response to PMA (results not shown). Ba^{2+} was not able to stimulate LH exocytosis in cells made refractory to Ca^{2+}, whereas Ba^{2+} could stimulate LH when added to cells which had been maintained at low $[Ca^{2+}]_{free}$ (pCa 7) (Fig. 5*b*). Ba^{2+}-stimulated LH exocytosis was half-maximal at pBa 3.6, a concentration 100-fold higher than the Ca^{2+} (Fig. 6). Concentrations of Ba^{2+} higher than pBa 3 inhibited LH exocytosis (Fig. 6) in a manner similar to the inhibition of LH exocytosis by very high Ca^{2+} concentrations (Fig. 6). Maximally effective concentrations of $[Ba^{2+}]_{free}$ (pBa 3) and $[Ca^{2+}]_{free}$ (pCa 5) stimulated LH exocytosis to the same extent [13.9 ± 1.5 and 13.8 ± 1.5% of LH released respectively (mean ± S.E.M., $n = 5$)]. Ba^{2+}-stimulated LH exocytosis, like Ca^{2+}-stimulated LH exocytosis, was not inhibited by staurosporine (Fig. 2). Taken together, these findings suggest that Ba^{2+} and Ca^{2+} stimulate exocytosis by the same mechanism and provide further evidence against a major role for protein kinase C in Ca^{2+}-stimulated LH exocytosis.

DISCUSSION

The aim of this study was to establish whether Ca^{2+} stimulates LH exocytosis by the activation of protein kinase C. McArdle *et al.* (1987) addressed this question indirectly, finding that protein kinase C down-regulation did not inhibit Ca^{2+}-ionophore (A23187)-stimulated LH exocytosis in intact rat anterior pituitary cells. However, protein kinase C can modulate the activity of several important Ca^{2+}-regulating proteins (Nishizuka, 1986; Yamaguchi *et al.*, 1987; Smallwood *et al.*, 1988; Yada *et al.*, 1989; Yoshida & Nachmias, 1989), which may affect the extent of ionophore-induced increases in intracellular $[Ca^{2+}]_{free}$. Furthermore, experiments involving protein kinase C down-regulation are difficult to interpret, for reasons outlined below. We have therefore used several approaches, in addition to protein kinase C down-regulation, to investigate the possible role of

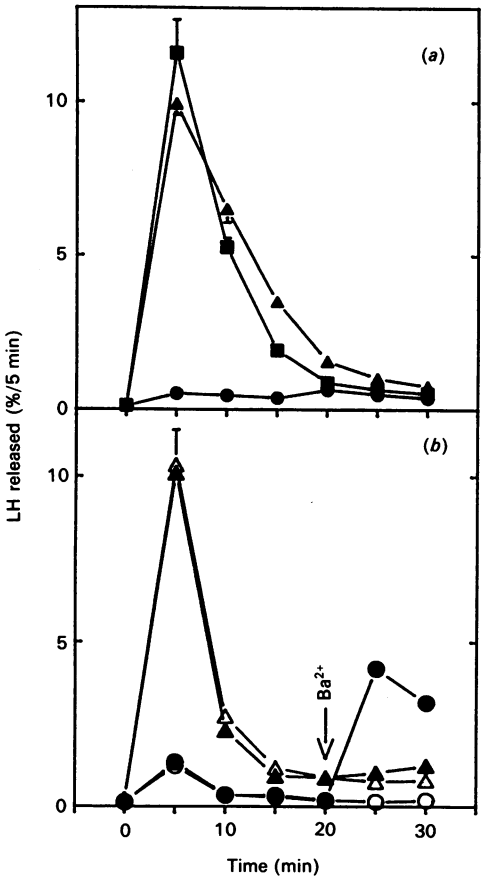

Fig. 5. (*a*) Time course of Ba^{2+}- and Ca^{2+}-stimulated LH exocytosis; (*b*) effect of Ba^{2+} on cells refractory to Ca^{2+}

In (*a*) permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with pCa 8 (●), pCa 5 (■), or pBa 3 (▲). LH exocytosis was initiated by replacing with identical buffer at 37 °C, which was exchanged at 5 min intervals. In (*b*) permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with pCa 7 (○, ●) or 5 (△, ▲). LH exocytosis was initiated by replacing with identical buffer at 37 °C, which was exchanged at 5 min intervals. BaEGTA (30 mM, pBa 3) was added from $t = 20$ min onwards (arrow) (▲, ●). In (*a*) and (*b*) the values at each time point represents the LH released during the preceding 5 min interval, and the $t = 0$ point represents the rate of LH release (per 5 min) during the cold equilibration period.

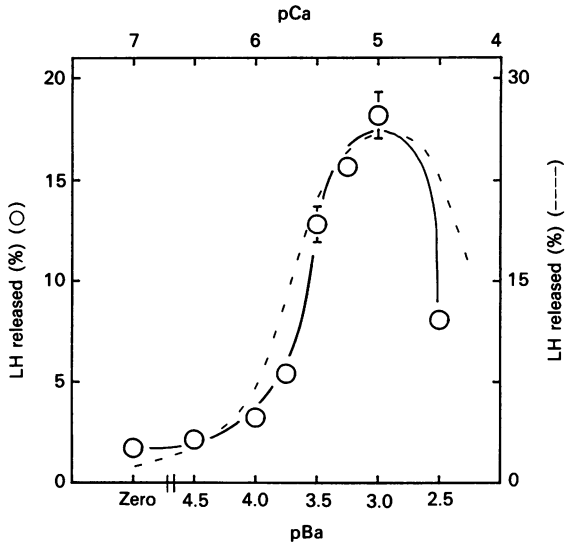

Fig. 6. Concentration-dependence of Ba^{2+}-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer with BaEGTA buffers giving the indicated $[Ba^{2+}]_{free}$ (○). LH exocytosis was initiated by replacing with identical medium at 37 °C, and LH released over the next 10 min was measured. Also shown is the concentration-dependence of Ca^{2+}-stimulated LH exocytosis (----) drawn from data in van der Merwe *et al.* (1989).

protein kinase C in Ca^{2+}-stimulated exocytosis in permeabilized cells, which allow direct control of the intracellular $[Ca^{2+}]_{free}$.

Several lines of evidence suggest that Ca^{2+}-stimulated LH exocytosis is not mediated by protein kinase C. Firstly, the protein kinase C activator PMA enhances LH exocytosis at all Ca^{2+} concentrations (van der Merwe *et al.*, 1989, and Figs. 2 and 4). If Ca^{2+} were acting via protein kinase C, LH exocytosis at maximal Ca^{2+} concentrations should not be further enhanced by PMA, since PMA does not enhance maximal Ca^{2+}-stimulated protein kinase C activity *in vitro* (Sekiguchi *et al.*, 1988). These results suggest that the protein kinase C activated by PMA in gonadotropes is largely Ca^{2+}-independent, which is in agreement with the finding that the major rat pituitary protein kinase C isoenzyme (type II or β) (Yoshida *et al.*, 1988) shows only slight Ca^{2+}-dependence when compared with the type I (γ) and III (α) isoenzymes (Sekiguchi *et al.*, 1988).

Secondly, PMA was able to stimulate LH exocytosis in cells made refractory to high $[Ca^{2+}]_{free}$. Two possible explanations for this finding are: (*a*) that there exists a pool of releasable LH which is sensitive to PMA, but not to Ca^{2+}; or (*b*) that PMA and Ca^{2+} stimulate LH release from the same store, but that the Ca^{2+} mechanism becomes desensitized before depletion of releasable LH occurs. Although our results cannot distinguish between these two possibilities, both these explanations imply that Ca^{2+} is not acting through protein kinase C.

Thirdly, the protein kinase C inhibitor staurosporine had little effect on Ca^{2+}-stimulated LH exocytosis, though it blocked PMA-stimulated LH exocytosis. Although staurosporine is a non-specific protein kinase C inhibitor, in that it also inhibits protein kinase A (Tamaoki *et al.*, 1986) and myosin light-chain kinase (Watson *et al.*, 1988), the absence of an inhibitory effect is strong evidence that Ca^{2+}-stimulated LH exocytosis is not mediated by protein kinase C.

Fourthly, Ca^{2+} stimulates LH exocytosis in cells completely desensitized to PMA. Although not demonstrated directly here, it is likely that this desensitization results from depletion of protein kinase C (Phillips & Jaken, 1983; Rodriguez-Pena & Rozengurt, 1984; McArdle *et al.*, 1987; Stojilkovic *et al.*, 1988). Experiments in which protein kinase C is depleted by prolonged phorbol ester stimulation are difficult to interpret for the following reasons. (*a*) Prolonged activation of protein kinase C by phorbol ester is likely to result in pleiotropic cellular changes, owing to phosphorylation of multiple proteins (Nishizuka, 1986). Thus observed changes may be due to mechanisms other than down-regulation of protein kinase C. (*b*) Prolonged exposure to phorbol ester results in depletion of LH stores, despite LH synthesis (McArdle *et al.*, 1987; Stojilkovic *et al.*, 1988; the present work), and this is likely to decrease the amount of releasable LH. It is not clear how one might correct for this effect (Stojilkovic *et al.*, 1988). Finally, (*c*) the rate of protein kinase C down-regulation varies between cell types (Adams & Gullick, 1989) and between different isoenzymes (Huang *et al.*, 1989; Kishimoto *et al.*, 1989). Together, these considerations suggest that experiments conducted on protein kinase C down-regulated cells should be interpreted with caution and should not be used as the only evidence for or against a role for protein kinase C in cellular processes.

Fifthly, in agreement with studies on catecholamine exocytosis using PC-12 cells (Mattheis *et al.*, 1988), Ba^{2+} was able to stimulate LH exocytosis, even though Ba^{2+} is a poor activator of protein kinase C *in vitro* (Takai *et al.*, 1979; Sekiguchi *et al.*, 1988), and in fact inhibits the type II or β isoenzyme (Sekiguchi *et al.*, 1988), which is the major isoenzyme present in the rat pituitary (Yoshida *et al.*, 1988). Our finding that PMA is able to stimulate LH exocytosis from cells refractory to Ba^{2+} also indicates that Ba^{2+} does not stimulate exocytosis by activating protein kinase C. The following findings suggest that Ba^{2+} and Ca^{2+} stimulate LH exocytosis by the same mechanism: (*a*) Ba^{2+} and Ca^{2+} stimulated LH exocytosis with the same time course; (*b*) maximal Ba^{2+}- and Ca^{2+}-stimulated LH exocytosis was the same; (*c*) Ba^{2+} was unable to stimulate LH exocytosis from cells made refractory to Ca^{2+}; (*d*) high concentrations of both Ba^{2+} and Ca^{2+} inhibited LH exocytosis; and (*e*) neither Ba^{2+}- nor Ca^{2+}-stimulated LH exocytosis was inhibited by staurosporine.

Although the evidence presented above argues strongly against a role for protein kinase C as the mediator of Ca^{2+}-stimulated LH exocytosis, this does not necessarily imply that Ca^{2+} and PMA stimulate exocytosis by completely separate and parallel pathways. The data are compatible with Ca^{2+} and PMA activating a final common exocytotic pathway distal to protein kinase C and the Ca^{2+} target. Although the identity of the Ca^{2+} target remains unknown, one possible candidate for which there is some evidence is the ubiquitous Ca^{2+}-binding protein calmodulin (Conn *et al.*, 1987). A role for calmodulin was suggested by the ability of anti-psychotic calmodulin inhibitors to inhibit Ca^{2+}-stimulated LH exocytosis (Conn *et al.*, 1981; Davidson *et al.*, 1987*a*). However, these hydrophobic agents lack specificity in that they have effects on surface membrane potential (McLaughlin & Whitaker, 1988), inhibit protein kinase C (Sanchez *et al.*, 1983), and bind to several non-calmodulin proteins in a Ca^{2+}-dependent way (Moore & Dedman, 1982). In agreement with these findings, we have found that the anti-psychotic phenothiazine trifluoperazine inhibits Ca^{2+}-, Ba^{2+}- and PMA-stimulated LH exocytosis in permeabilized cells with equal potency (P. A. van der Merwe & J. S. Davidson, unpublished work), and thus appears to be a non-specific inhibitor of exocytosis. Our finding that Ba^{2+} can stimulate LH exocytosis argues strongly against a role for calmodulin in Ca^{2+}-stimulated LH exocytosis, since Ba^{2+} does not bind to calmodulin or activate calmodulin-dependent enzymes even at concentrations as high as 1 mM (Chao *et al.*, 1984; Kuret & Schulman, 1984). In support of this, we have found that calmidazolium, a much more potent and specific calmodulin inhibitor than trifluoperazine (Gietzen *et al.*, 1981), does not inhibit Ca^{2+}-stimulated LH exocytosis (P. A. van der Merwe & J. S. Davidson, unpublished work).

The opposite conclusion about the target for Ca^{2+} in exocytosis has been reached in studies on permeabilized bovine adrenal chromaffin cells (Burgoyne *et al.*, 1988; Knight *et al.*, 1988). Burgoyne *et al.* (1988) demonstrated moderate inhibition of Ca^{2+}-stimulated exocytosis in protein kinase C down-regulated cells, and concluded that protein kinase C has a major role in Ca^{2+}-stimulated exocytosis. Knight *et al.* (1988) tested a range of protein kinase C inhibitors (but not staurosporine) and found none that did not also inhibit Ca^{2+}-stimulated exocytosis. However, these results may simply reflect a lack of specificity of the agents used. They also found that exocytosis and protein kinase C (purified from bovine adrenal medulla) had similar cation and nucleotide specificities (Knight *et al.*, 1988). Although these data are consistent with a role for protein kinase C in exocytosis, they do not constitute proof of one.

Our findings suggest that protein kinase C is not a mediator of the acute LH exocytosis stimulated by a GnRH-induced increase in intracellular $[Ca^{2+}]_{free}$. Although the precise role of protein kinase C is not known, there is some evidence that it mediates (*a*) GnRH stimulation of LH β-subunit gene transcription and (*b*) the modulatory effects of GnRH on the GnRH receptor (Conn, 1989).

In conclusion, our results indicate that Ca^{2+} stimulates LH exocytosis by a mechanism which does not involve the activation of protein kinase C. Our results also argue against a role for calmodulin in Ca^{2+}-stimulated LH exocytosis. Although our data do not suggest what the Ca^{2+} target is, there are a large number of Ca^{2+}-sensitive proteins which have already been identified which may mediate Ca^{2+}-stimulated exocytosis (Weeds, 1982; Martin & Creutz, 1987; Suzuki, 1987; Pollard *et al.*, 1988; Burgoyne & Geisow, 1989; Martin & Walent, 1989).

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