Sensitization of regulated exocytosis by protein kinase C

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Activation of protein kinase C (PKC) increases vesicular secretion in many cell types. We determined the calcium dependence of secretion and the size of the readily releasable pool of secretory granules in pituitary gonadotropes by photorelease of caged-calcium. The calcium affinity for exocytosis was roughly doubled by activation of PKC by a phorbol ester, whereas the size of the readily releasable pool was not greatly increased. The effect was due to activation of PKC, because it was blocked by a PKC inhibitor and was not mimicked by an inactive phorbol ester analogue. A similar increase in calcium sensitivity was induced by preincubation with gonadotropin-releasing hormone, the physiological releasing hormone. These findings provide direct evidence for physiological regulation of secretion by enhancement of Ca^{2+}-sensing steps. Because exocytosis depends on the third- to fourth-power of intracellular free Ca^{2+} concentration, this mechanism ensures a powerful up-regulation of hormone release and may explain how PKC can stimulate exocytosis without an increase of Ca^{2+} above the resting level.

PKC | Ca^{2+} sensitivity | vesicle pools

Neurons and endocrine cells release neurotransmitters and hormones by highly regulated exocytosis of secretory vesicles or granules. Factors implicated in this regulation include Ca^{2+}, protein kinase C (PKC), protein kinase A, calmodulin, Munc13-1, and others (1–5). Here we focus on regulation by PKC. Activation of PKC by phorbol esters or diacylglycerols enlarges the readily releasable pool (RRP) of vesicles in chromaffin cells (3) and in hippocampal neurons (6). On the other hand, less direct work suggests that activation of PKC may increase the calcium sensitivity of secretion rather than RRP size in chick ciliary ganglion (7) and the calyx of Held (8). We now provide direct measurements supporting this latter conclusion in pituitary gonadotropes.

Intracellular Ca^{2+} is the principal regulator of stimulus–secretion coupling in many types of cells (9, 10), but there are examples of Ca^{2+}-independent secretion (11), i.e., secretion that does not require elevated levels of Ca^{2+}. They include secretion from epithelial cells stimulated by activating PKC or protein kinase A (12, 13), from mast cells dialyzed intracellularly with guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) (14, 15), or from insulin-secreting cells activated through latrophilin (16). Similarly, in pituitary gonadotropes, the resting secretion rate can be increased 10-fold by applying phorbol-12-myristate-13-acetate (PMA, an activator of PKC) with no detected Ca^{2+} elevation (17). In these cases, where exocytosis can be initiated independent of Ca^{2+}, Ca^{2+} alone can trigger exocytosis as well. An important question then is whether or not these various triggering molecules are just increasing the Ca^{2+} sensitivity of exocytosis so that significant membrane fusion occurs even at resting intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) levels. The normal steep dependence of exocytosis on $[Ca^{2+}]_i$ means that even a small change in Ca^{2+} sensitivity could have large effects on the overall rate. Our work suggests that this model applies for gonadotropes.

Materials and Methods

Cell Culture and Solutions. Isolated gonadotropes were prepared as described (18). The anterior pituitary was removed from 4- to 6-week-old male Sprague–Dawley rats that had been castrated at week 2 by the local supplier. Cells were used 2–4 days after dispersion. Experiments were done at 20–24°C in a bath solution containing 140 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 10 mM glucose (pH 7.4). At the end of each experiment, we confirmed that we were recording from a gonadotrope by challenging the cell with gonadotropin-releasing hormone (GnRH, 5 nM) and verifying a rapid, hormonally induced increase of $[Ca^{2+}]_i$.

Membrane Capacitance (C_m) Measurement. Conventional whole-cell recordings used sylgard-coated 2–3 MΩ pipettes. Series resistance ranged from 4 to 12 MΩ. An EPC-9 patch-clamp amplifier was used together with PULSE+LOCK-IN software. A 977-Hz, 20-mV peak-to-peak sinusoidal voltage stimulus was superimposed on a holding potential of −80 mV. Currents were filtered at 2.9 kHz and sampled at 15.6 kHz.

Generation and Measurement of $[Ca^{2+}]_i$ Ramps. To determine the $[Ca^{2+}]_i$ sensitivity of exocytosis, ramp $[Ca^{2+}]_i$ increases were generated by 10-s steady UV (380 nm) illumination (TILL Monochromator, TILL Photonics, Gräfelting, Germany) of caged-Ca^{2+}, nitrophenyl-EGTA (NP-EGTA). The same light was also used to monitor $[Ca^{2+}]_i$ increases by the low-affinity Ca^{2+} indicator, fura-6F. $[Ca^{2+}]_i$ was calculated as follows: $[Ca^{2+}]_i = K_d \times (F_{max} - F)/(F - F_{max}/\beta)$, where $\beta = F_{max}/F_{min}$ and was determined to be 13.3 in our system. K_d of fura-6F is 5.3 μM as published by Molecular Probes. Because the basal $[Ca^{2+}]_i$ is buffered at ≈200 nM, far below the K_d of fura-6F, the initial fluorescence value can be used as $F_{max.}$ We neither measured the apparent K_d of fura-6F under our conditions nor compensated for photobleaching.

Flash Photolysis. Flashes of UV light and fluorescence-excitation light were generated as described (19) except that we used a TILL flash lamp. The NP-EGTA-containing internal solutions consisted of 110 mM CsCl, 5 mM NP-EGTA, 2 mM NaCl, 4 mM $CaCl_2$, 2 mM MgATP, 0.3 mM GTP, 0.2 mM fura-6F, and 35 mM Hepes. Its basal $[Ca^{2+}]_i$ was measured to be ≈200 nM by fura-2. Pipette solutions were adjusted to pH 7.2 with HCl or CsOH. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio (20). All experiments were performed at room temperature. To get $[\Delta C_m/\Delta t]_{max}$, we divided the change of capacitance at time $\tau/2$ by $\Delta t = \tau/2$, where τ is the time constant from the exponential fit of the burst.

Abbreviations: RRP, readily releasable pool; PMA, phorbol-12-myristate-13-acetate; C_m, membrane capacitance; GnRH, gonadotropin-releasing hormone; NP-EGTA, nitrophenyl-EGTA; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; 4α-PDD, 4α-phorbol-12,13-didecanoate; BIS, bisindolylmaleimide.

See commentary on page 16522.

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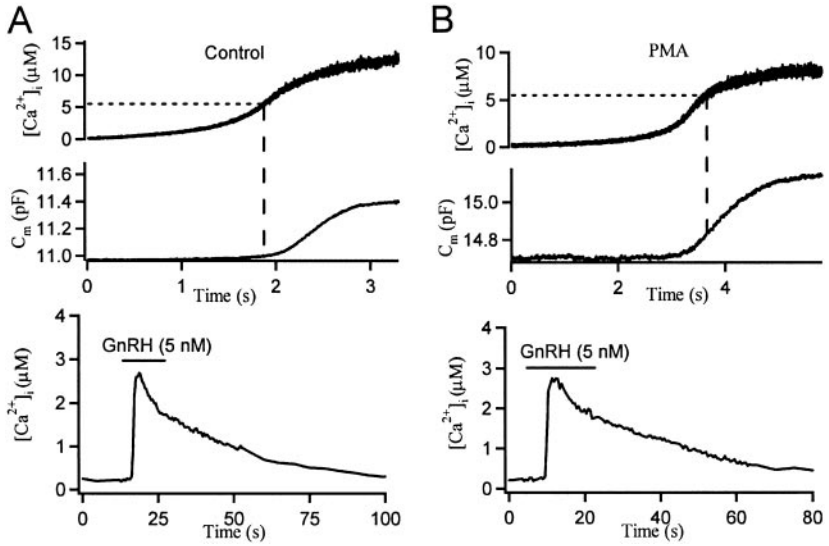

Fig. 1. PMA lowers the calcium threshold for secretion in gonadotropes. Steady UV illumination generates ramp $[Ca^{2+}]_i$ increases with intracellular caged Ca^{2+} and measures $[Ca^{2+}]_i$ by using a Ca^{2+} indicator, fura-6F. Simultaneous time courses of $[Ca^{2+}]_i$ and C_m for a single control gonadotrope (*A*) and for a PMA-treated (for 2–3 min) gonadotrope (*B*). Note at the same $[Ca^{2+}]_i$ level, C_m only begins to increase in the control cell, whereas it already takes off in the PMA-treated cell. At the end of the experiments, the cells were challenged by 5 nM GnRH to verify them as gonadotropes (*Lower*).

Results

Identifying mechanisms for stimulation of exocytosis by PKC is complicated by the range of possible targets that may contribute in concert. Using pituitary gonadotropes, we simplified the task by photoreleasing intracellular Ca^{2+} from a caged-Ca^{2+} compound as the stimulus. By simultaneously monitoring Ca^{2+}, with optical indicators, and exocytosis, using C_m as a measure of plasma membrane area, we could measure the size of the RRP and the Ca^{2+} sensitivity of exocytosis directly. Our first experiments used continuous UV illumination to uncage and ramp up $[Ca^{2+}]_i$ slowly, giving in effect an intracellular Ca^{2+} titration of exocytosis. Because the RRP is released much faster than it is refilled, we focused on the initial period of rapid exocytosis. The steady UV illumination released Ca^{2+} from caged-Ca^{2+} (NP-EGTA) continuously, increasing $[Ca^{2+}]_i$ gradually by $\approx$10 μM within seconds (Fig. 1*A*). The plasma membrane area (recorded as C_m) remained constant at the beginning when $[Ca^{2+}]_i$ was low but at a certain point took off rapidly, adding 0.2–0.4 pF (20–40 μm^2) of membrane as $[Ca^{2+}]_i$ was rising. Then with depletion of the RRP, the change of C_m slowed to a rate that was limited by the resupply of secretory granules to the RRP. In the control cell shown, C_m just begins to rise at a $[Ca^{2+}]_i$ of 5.5 μM (Fig. 1*A*, dashed line), whereas in the cell pretreated with 100 nM PMA, C_m is already in the middle of its rapid rising phase. To confirm that we were recording from gonadotropes, we challenged cells with GnRH (5 nM), at the end of the experiment, producing a rapid hormonally induced increase of $[Ca^{2+}]_i$ (Fig. 1 *Lower*). This increase was not the normal oscillatory response of gonadotropes because the internal solution contained 5 mM NP-EGTA, with a very high buffering capacity. In fura-2-AM loaded cells without NP-EGTA, we did observe GnRH-induced $[Ca^{2+}]_i$ oscillations (data not shown).

To quantify the effects of PMA, we took the $[Ca^{2+}]_i$ level at the half-maximal rate of exocytosis as our index of the calcium sensitivity of exocytosis, where the rate was the slope of a polynomial fitted to the C_m traces (Fig. 2*A*). We also fitted a straight line to the last slow phase of the C_m increase and extrapolated it back to the time when the half-maximal rate was reached. The difference (ΔC_m) between this extrapolated level and the baseline C_m was our measure of RRP size. By using a kinetic model of exocytosis that requires three Ca^{2+}-binding

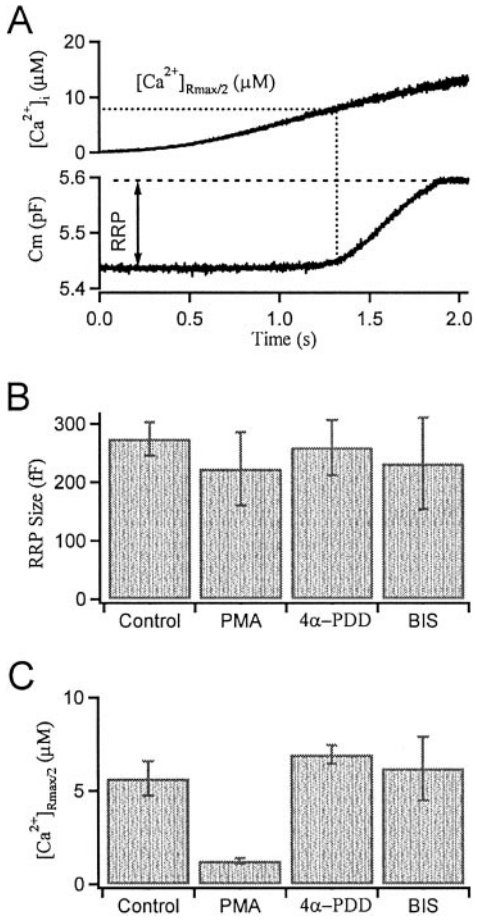

Fig. 2. Summary of PMA actions on exocytosis in gonadotropes. (*A*) Simultaneous time courses of $[Ca^{2+}]_i$ and C_m for a single control gonadotrope illustrating how the RRP size and the $[Ca^{2+}]_i$ threshold are determined by ramp $[Ca^{2+}]_i$ experiments. (*B*) Pretreatments with PMA (100 nM), 4α-PDD (1 μM), and BIS (500 nM) have no significant effect on the RRP size (defined in text as ΔC_m). (*C*) The calcium threshold (measured as $[Ca^{2+}]_{Rmax/2}$) is lowered by PMA, but not by 4α-PDD or by PMA in combination with BIS.

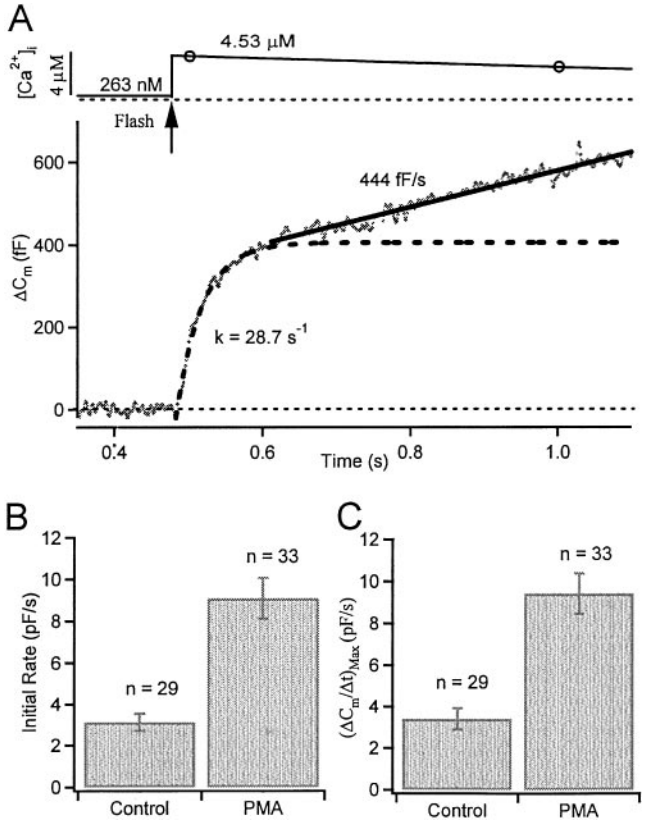

Fig. 3. PMA increases the initial rate of exocytosis in gonadotropes. (*A*) Simultaneous measurements of $[Ca^{2+}]_i$ level and membrane area C_m after a UV flash (arrow) uncages Ca^{2+} within 2 ms. Open circles are measured calcium points (continuously measured every 0.5 s). C_m increases in an early burst (fitted with single exponential with the rate constant, $k = 1/\tau$ indicated) followed by a sustained component (fitted with a straight line of the indicated slope). (*B* and *C*) PMA increases the initial rate of secretion (measured 20 ms after the flash) and the maximum rate.

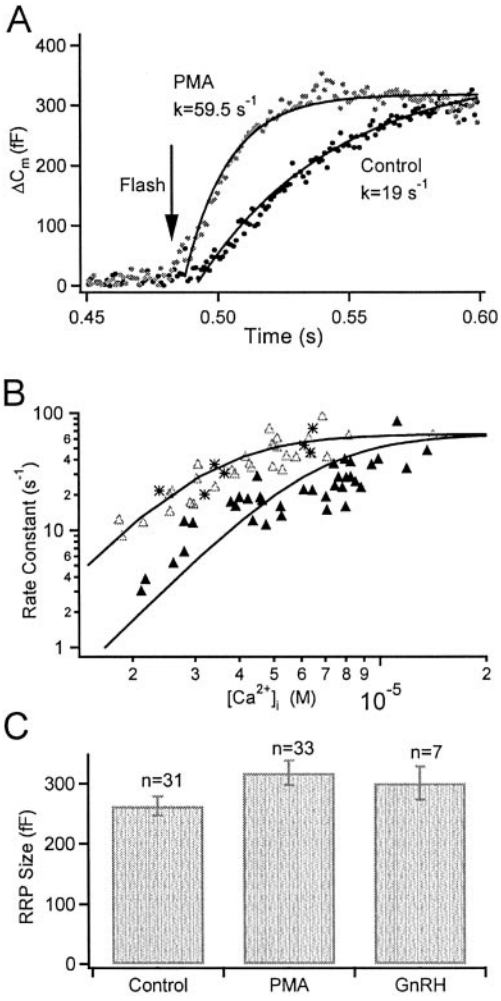

Fig. 4. PMA facilitates exocytosis in gonadotropes by increasing the calcium sensitivity of release without significantly altering RRP size. (*A*) Averaged C_m responses for control (filled symbols) and PMA (100 nM, open symbols) treated gonadotropes, summarizing experiments that had postflash $[Ca^{2+}]_i$ values between 4 and 6 μM. Superimposed curves are single exponential fits with the rate constants indicated. (*B*) PMA increases the Ca^{2+} sensitivity of release (all experiments plotted individually). Rate constants for exponential fits of the exocytotic burst are plotted against calcium levels for control (filled triangles), PMA-treated (open triangles, 100 nM for 2–3 min), and GnRH-treated (asterisks, 5 nM for 2 min) gonadotropes, respectively. The continuous curves represent the equation: Rate $= R/(1 + (K_d/[Ca^{2+}]_i)^3)$. (*C*) RRP size is unchanged by PMA or GnRH preincubation.

steps before fusion (21), computer simulation demonstrated that this method of back extrapolation approximates the real RRP size within 10–20 fF (1–2 μm^2). Unexpectedly, PMA did not increase the RRP size measured this way (Fig. 2*B*). Instead it greatly reduced the $[Ca^{2+}]_i$ level needed to evoke a half-maximal rate of exocytosis ($[Ca^{2+}]_{Rmax/2}$) (from 5.66 ± 0.92 μM, mean ± SEM, in control gonadotropes to 1.29 ± 0.12 μM; Fig. 2*C*). This effect of PMA was not mimicked by 4α-phorbol-12,13-didecanoate (4α-PDD) (1 μM) and was blocked by the PKC inhibitor bisindolylmaleimide (BIS) (500 nM in both the bath and the patch pipette). The RRP size was not affected by 4α-PDD or BIS.

To validate conclusions drawn from our calcium-ramp experiments, we used flash photolysis of caged-Ca^{2+} to raise Ca^{2+} in a step-like manner. The Ca^{2+} step elicits a burst of exocytosis followed by a slow continuous component (Fig. 3*A*). We fitted the initial time course of the exocytotic burst by a second-order polynomial and took the slope of this line 20 ms after the flash as our measure of the initial rate of exocytosis (22). This initial rate tripled with 100 nM PMA (control, 3.13 ± 0.41 pF/s; PMA, 9.12 ± 0.97 pF/s; Fig. 3*B*), despite a smaller mean postflash $[Ca^{2+}]_i$ level compared with controls (control, 5.73 ± 0.42 μM; PMA, 4.22 ± 0.27). Similarly, the maximum rate of secretion was increased (control, 3.40 ± 0.51 pF/s; PMA, 9.40 ± 0.96 pF/s; Fig. 3*C*).

To compare the kinetics of exocytosis at similar $[Ca^{2+}]_i$ levels, we averaged burst responses for experiments with postflash $[Ca^{2+}]_i$ values (measured 20 ms after the flash) between 4 and 6 μM (mean $[Ca^{2+}]_i$ values: control, 4.77 ± 0.19 μM, n = 10; PMA, 4.72 ± 0.16 μM, n = 15). The exocytotic burst of chromaffin cells must be fitted by two exponentials (23), but for gonadotropes it is reasonably fitted by one (Fig. 4*A*). The burst is speeded by PMA (in Fig. 4*A* the rate constant increases from 19 s^{-1} to 60 s^{-1}). Plotting the rate constants against postflash $[Ca^{2+}]_i$ levels gives the calcium dependence of fusion of the RRP, which shifts toward lower Ca^{2+} concentrations with PMA (Fig. 4*B*). The curves are the equation: Rate $= R/(1 + (K_d/[Ca^{2+}]_i)^3)$, where R is the maximum rate and K_d is the $[Ca^{2+}]_i$ level where the rate reaches the half maximum. Restraining R to a common saturating rate, the fitted K_d were 6.71 ± 0.36 μM (control) and 3.39 ± 0.20 μM (PMA), consistent with the Ca^{2+}-ramping experiments. Extrapolating the fitted curves predicts that PMA treatment would increase the release rate by 7.7-fold at the 100–200 nM resting $[Ca^{2+}]_i$ level, in agreement with our previous

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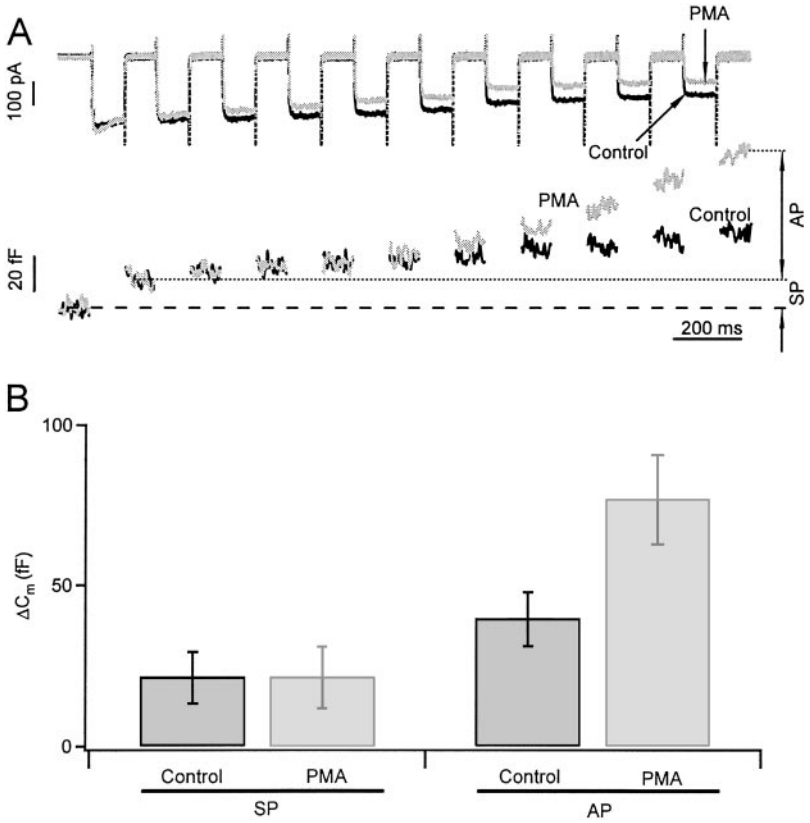

Fig. 5. PMA augments the size of the asynchronous pool (AP) but not the synchronous pool (SP) of secretory granules. (*A*) Calcium currents and C_m response to a train of ten 100-ms depolarizing pulses from −70 to 0 mV. Traces displayed are from the same cell, before and 3 min after PMA treatment. The change of C_m in response to the first depolarization defines the SP size and the subsequent increase in C_m defines the AP. PMA increased AP by a factor of 2 (despite a reduction on calcium currents), but left SP unaltered. (*B*) Summary of the effect of PMA on SP and AP from six cells.

observations that PMA induces spontaneous secretion from gonadotropes (17).

In contrast to many other excitable cells, in gonadotropes the physiological stimulus for secretion is usually not depolarization. Gonadotropes normally secrete in response to GnRH, which induces intracellular Ca^{2+} release via the phospholipase C and inositol trisphosphate pathway (24). Nevertheless, we were able to demonstrate the effect of PMA on exocytosis induced by depolarizing stimuli (Fig. 5). We used whole-cell patch clamp to monitor calcium channel current and C_m simultaneously. Trains of depolarizations elicited calcium influxes and small C_m increases. An initial small increase of C_m (≈20 fF) occurring within the first 100-ms depolarization to 0 mV probably corresponds to the fusion of readily releasable secretory granules close to the calcium channels. We will call these granules the synchronous pool. A subsequent, much slower increase of C_m may correspond to readily releasable granules that are farther away from calcium channels. We will call those granules the asynchronous pool. PMA (100 nM) treatment increased the amplitude of the asynchronous pool by a factor of 2.1 without altering that of the synchronous pool (Fig. 5). The increase of the asynchronous pool was not due to more calcium entry, because PMA actually decreased the calcium currents slightly (see Fig. 5*A*). However, the increase of the asynchronous pool with no change of the synchronous pool would be consistent with the following hypothesis: The RRP has not increased and the number of granules docked near calcium channels has not changed, but because the calcium sensitivity of the RRP has increased, the calcium spreading from each open calcium channel has a larger effective domain of action and can trigger fusion of more distant granules.

GnRH, the natural secretagogue for gonadotropes, not only generates IP_3 and Ca^{2+} elevations, but also activates PKC. Is this PKC activation physiologically relevant for sensitizing exocytosis? Indeed, 2-min pretreatments with GnRH (5 nM) increased the rate of the flash-induced exocytotic bursts (asterisks, Fig. 4*B*). Neither GnRH nor PMA affected RRP size (Fig. 4*C*).

Discussion

We provide direct evidence that activating PKC leads to a 2-fold rise in the apparent Ca^{2+} affinity of the exocytotic machinery without noticeable effect on the RRP size in gonadotropes. Previous studies of the chick ciliary ganglion synapse (7) and of the rat calyx of Held (8) suggested the same conclusion in those cells by using less direct methods. In contrast, PKC increases the RRP size in chromaffin cells (3) and hippocampal neurons (6). In addition, PKC activation increases the Ca^{2+} sensitivity of exocytosis in chromaffin cells by increasing the percentage of vesicles that are in a highly Ca^{2+}-sensitive state (25). It is tempting to speculate how PKC activation increases the Ca^{2+} sensitivity for secretion in gonadotropes. In one hypothesis, the Ca^{2+} sensor of gonadotropes contains some isoform of synaptotagmin whose Ca^{2+} sensitivity can be modulated by phosphorylation. Alternatively, considering the calcium sensor and the soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) complex as an integrated fusion machine (26), one could envision that the fusion machine in gonadotropes is held in a low-affinity state by certain clamping proteins. Phosphorylation of the clamping proteins might release the clamp and sensitize secretion. It would be of great interest to know the nature of PKC action on the Ca^{2+} sensitivity of fusion and to identify the substrates of PKC.

It is somewhat puzzling that PKC has no effect on RRP size. As suggested in previous work, PKC increases the RRP size in chromaffin cells and hippocampal neurons. This effect has been attributed to the facilitation of a recruitment step to refill RRP. Speeding of refilling seems equally necessary in gonadotropes. Because the Ca^{2+} sensitivity of release is increased by PKC, the spontaneous release rate at resting $[Ca^{2+}]_i$ should be profoundly facilitated as we expect from the cubic law of Ca^{2+} dependence of exocytosis. If the rate of RRP refilling were not increased as well by PKC, the RRP would be expected to shrink, which is not the case in our experiments. Thus, PKC seems to act by two distinctive mechanisms to modulate exocytosis: first, by accelerating the refilling of the RRP, possibly in all cell types, and second, by sensitizing the Ca^{2+} sensor of the exocytotic machinery in a few cell types.

The finding that PKC sensitizes the exocytotic machinery to Ca^{2+} provides an explanation for Ca^{2+}-independent stimulation–secretion coupling in various cells (11): secretion without a calcium elevation. The 2-fold increase in the Ca^{2+} sensitivity of exocytosis that we find predicts an 8-fold increase in the extrapolated release rate at resting $[Ca^{2+}]_i$ level, similar to what we have previously reported in gonadotropes (13). Similar direct measures of the Ca^{2+} sensitivity of release could now be applied to other systems where there is Ca^{2+}-independent stimulation of secretion. In gonadotropes, sensitization of exocytosis by PKC should increase the efficacy of GnRH-stimulated secretion of gonadotropins from the pituitary during physiological GnRH pulses, which last 2–6 min (27). Sensitization may also extend the period of secretion beyond the time when the cytoplasmic Ca^{2+} falls to near resting levels.

It has been demonstrated that the synchronous pool (also called the immediately releasable pool) of granules constitutes only a small fraction ($\approx$10%) of the RRP in gonadotropes (this study) and in other endocrine cells including adrenal chromaffin cells (28) and pancreatic beta cells (29). Because the vast majority of the RRP is far away from the calcium channels, any mechanism that modulates the release probability of these granules could have great importance in regulating hormone release. Enhancing the Ca^{2+} sensitivity of RRP would enable exocytosis at sites relatively distant from Ca^{2+} channels at intermediate or resting $[Ca^{2+}]_i$ levels, and it may be a widespread pathway for secretory control.

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