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Heterogeneity of the Ca^{2+} Sensitivity of Secretion in a Pituitary Gonadotrope Cell Line and its Modulation by Protein Kinase C and Ca^{2+}

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Modulation of the Ca^{2+} sensitivity and cooperativity of secretion is an important means of regulating neurotransmission and hormone secretion. Employing high-time resolution measurement of membrane capacitance (Cm) stimulated by step-like or ramp $[Ca^{2+}]_i$ elevation, we have identified the co-existence of both a high and low Ca^{2+}-sensitive exocytosis in an immortal pituitary gonadotrope cell line, LβT2. Ramp $[Ca^{2+}]_i$ generated by slow uncaging elicited a biphasic C_m response. The first phase of response, which represents a highly Ca^{2+}-sensitive pool (HCSP) of vesicles, began to secrete at low $[Ca^{2+}]_i$ concentration (<1 μM) with low Ca^{2+} cooperativity. In contrast, the second phase, which represents a lowly Ca^{2+}-sensitive pool (LCSP) of vesicles, only exocytozed at higher $[Ca^{2+}]_i$ (>5 μM) and displayed a steep Ca^{2+} cooperativity. The co-existence of vesicle populations with different Ca^{2+} sensitivities was further confirmed by flash photolysis stimuli. The size of the HCSP was ~30 fF under resting conditions, but was dramatically increased (~ threefold) by application of phorbol-12-myristate-13-acetate (PMA, an activator of protein kinase C). Forskolin (an activator of protein kinase A), however, exerted no significant effect on the size of both HCSP and LCSP. GnRH (gonadotropin releasing hormone) augmented the size of both pools to a larger extent (5- and 1.7-fold increase for HCSP and LCSP, respectively). The heterogeneity of Ca^{2+} sensitivity from different pools of vesicles and its differential modulation by intracellular signals suggests that LβT2 cells are an ideal model to further unravel the mechanism underlying the modulation of Ca^{2+}-sensing machineries for exocytosis. J. Cell. Physiol. 207: 668–674, 2006. © 2006 Wiley-Liss, Inc.

Neurons and endocrine cells release neurotransmitters and hormones by highly regulated exocytosis of synaptic vesicles or secretory granules. The existence of vesicles in different maturation steps results in different time requirements for release. Consequently, multiple phases of release were observed upon prolonged stimulation (Neher and Zucker, 1993; Thomas et al., 1993; Tse et al., 1997): an initial rapid phase (often termed the "exocytotic burst") and a slower, sustained phase. It is generally held that the exocytotic burst represents the release of a pool of vesicles that are "readily releasable" (see Sorensen (2004) for a recent review). Detailed analysis of the exocytotic burst from chromaffin cells has revealed a sum of two exponentials, indicating it can be further divided into two separate pools: the readily releasable pool (RRP, release kinetics with a rate constant of 25–50 sec^{-1} at 20 μM $[Ca^{2+}]_i$), and the slowly releasable pool (SRP, release kinetics with a rate constant of 5 sec^{-1} at 20 μM $[Ca^{2+}]_i$) (Voets et al., 1999; Sorensen, 2004). It has been suggested that the two pools are arranged sequentially since refilling of the RRP after depletion coincides with a decrease in the SRP (Voets et al., 1999). However, a parallel arrangement of the two pools has also been proposed (Takahashi et al., 1997).

Recently, we and others have revealed a highly Ca^{2+}-sensitive pool (HCSP) of vesicles in pituitary gonadotropes (Zhu et al., 2002; Yang et al., 2005). The HCSP has much faster release kinetics at low $[Ca^{2+}]_i$ even when compared with the RRP in chromaffin cells. The release from the HCSP has a shallow cooperativity on $[Ca^{2+}]_i$ (Yang and Gillis, 2004; Yang et al., 2005), whereas release from the RRP has previously been shown to depend on $[Ca^{2+}]_i$ raised to the 3rd–5th power (Thomas et al., 1993; Heinemann et al., 1994; Barg et al., 2001). The higher Ca^{2+} sensitivity and shallow Ca^{2+} cooperativity render the HCSP with the highest release probability during small global increases in $[Ca^{2+}]_i$. More interestingly, although very small in size under basal conditions, the HCSP can be augmented much more than the RRP by activation of PKC, indicating that HCSP may play an important role in physiological

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regulation of hormone secretion. The relationships between the RRP, SRP, and the HCSP have not yet been elucidated. For instance, it is not known if the HCSP is refilled from the RRP or if it represents distinct vesicles with different Ca^{2+}-sensor(s) for exocytosis. It is also intriguing to further uncover the underlying molecular mechanism by which PKC augments the HCSP. However, the large heterogeneity of anterior pituitary primary culture and the lower availability of gonadotropes (~ 5%) (Kaftan et al., 2000) in this culture limit detailed exploration of the molecular players. It is thus advantageous to employ an immortal gonadotrope cell line (LβT2) generated by Mellon and co-workers (Mellon et al., 1991). A previous study has revealed that LβT2 is a reasonable model to study the Ca^{2+} and secretory responses to GnRH (Thomas et al., 1996). In this study, we further characterized the properties of secretory pools of vesicles in LβT2 cells by employing time-resolved membrane capacitance measurement and Ca^{2+} uncaging techniques. We also explored how these different pools of vesicles might be regulated by GnRH and protein kinase C (PKC).

MATERIALS AND METHODS

Cell culture

The mice pituitary gonadotrope cell line LβT2 was kindly provided by Dr. Pamela L. Mellon from UCSD. The cells were grown in DMEM (Gibco, Carlsbad, CA) supplement with 10% FBS (Gibco) and 200 μM kanamycin sulfate (Sigma, St. Louis, MO) at 37°C gassed with a humidified mixture of 5% CO_2 and 95% air. Cells were passaged at about 1:3 by treatment of trypsin/EDTA (Sigma) once per week. Cells cultured for 2–4 days were used in the experiments. All measurements were performed at room temperature.

Membrane capacitance (C_m) measurement

The C_m of LβT2 cells was measured in real time using an EPC9 amplifier (Heka Electronics, Lambrecht, Germany) in conventional whole-cell patch clamp configuration. A sine + DC protocol was applied using the Lockin extension of the Pulse program (Heka Electronics). LβT2 cells were voltage clamped at a holding potential of −70 mV and a sine wave voltage command with amplitude of 20 mV and frequency of 977 Hz was applied. Currents were filtered at 2.9 kHz and sampled at 15.6 kHz.

The normal bath solution contained (in mM): 140 NaCl, 2.5 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (adjusted to pH 7.4 with NaOH, 308 mOsm). PMA (100 nM), forskolin (10 μM), or GnRH (10 nM) were applied by local perfusion with the micropipette pointing at cells started immediately after establishment of the whole-cell configuration and lasted throughout the recording process. Ca^{2+} uncaging and C_m measurement were initiated 2–3 min after drug treatment. To ensure recording from cells that have not previously encountered the drugs, cells were plated on a small square coverglass (roughly 5 × 5 mm). The coverglass was transferred to the recording chamber and only one cell was recorded for each coverglass. After the recording, the coverglass was removed and the recording chamber was washed three times with ethanol followed by at least three times with distilled water. We then added fresh bath solution to the chamber and started a new recording. Gö6976 (500 nM) or Heparin (1 mg/ml) was included in the pipette solution for blocking PKC activation and Ca^{2+} release form IP_3-sensitive intracellular stores, respectively. In addition, Gö6976 (500 nM) was also applied extracellularly by local perfusion. Unless otherwise stated, all reagents were purchased from Sigma.

Ca^{2+}-uncaging and $[Ca^{2+}]_i$ measurement

Homogenous global $[Ca^{2+}]_i$ elevation was generated by photolysis of the caged-Ca^{2+} compound, nitrophenyl-ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (NP-EGTA, Molecular Probes, Carlsbad, CA), with UV light source as previously described (Xu et al., 1997). The NP-EGTA containing pipette solution contained (in mM): 110 CsCl, 5 NP-EGTA, 2 NaCl, 4 $CaCl_2$, 2 MgATP, 0.3 GTP, 0.2 fura-6F, and 35 HEPES, adjusted to pH 7.2 using CsOH or HCl (osmolarity, 300 mOsm). Unless otherwise indicated, the free Ca^{2+} concentration of the pipette solution was determined to be around 200 nM.

$[Ca^{2+}]_i$ was measured using either the single wavelength or dual wavelength excitation method as suggested by Grynkiewicz et al. (1985). Single wavelength $[Ca^{2+}]_i$ determination was adopted in the so-called ramp Ca^{2+} stimulation where $[Ca^{2+}]_i$ is slowly increased in a ramp-like manner by photolysis of NP-EGTA using 380 nm UV illumination generated from a monochromator (Till Photonics, Planegg, Germany). The same light was also used to monitor $[Ca^{2+}]_i$ concentration by a low-affinity Ca^{2+} indicator, fura-6F (Molecular Probes). $[Ca^{2+}]_i$ was calculated as follows: $[Ca^{2+}]_i = K_d * (F_{max} - F)/(F - F_{max}/\beta)$, where $\beta = F_{max}/F_{min}$ (18). K_d of fura-6F is set to 5.3 μM as published by Molecular Probes. Because the basal $[Ca^{2+}]$ is buffered at ~200 nM, far below the K_d of fura-6F, the initial fluorescence value can be used as F_{max} (Xu and Bajjalieh, 2001; Zhu et al., 2002) We measured neither the apparent K_d of fura-6F under our experimental conditions, nor compensated for photobleaching. The extent of photobleaching over 3 seconds was estimate to be only 0.34 ± 0.14% upon continuous illumination of 380 nm UV light. Hence the influence of photobleaching on $[Ca^{2+}]_i$ measurement can be neglected.

The dual wavelength excitation method was used for $[Ca^{2+}]_i$ determination in a flash photolysis experiment in which the step-like $[Ca^{2+}]_i$ elevation was elicited by UV flash generated from a Rapp flash lamp (Rapp Optoelektronik, Hamburg, Germany). The flash was followed by a series of illuminations alternating between 350 and 380 nm, which allowed ratiometric determination of the Ca^{2+} concentration according to the equation (Grynkiewicz et al., 1985): $[Ca^{2+}]_i = K_{eff} \times (R - R_{min})/(R_{max} - R)$, where K_{eff}, R_{min}, and R_{max} are constants obtained from in vivo calibration. The duration of these illuminations was adjusted to maintain relatively constant Ca^{2+} concentrations, since the illumination at 350 and 380 nm also lead to photolytic release of Ca^{2+}. Trains of light alternating at 350 and 380 nm were generated from the monochromator (Till Photonics). The resulting fluorescence was acquired by a photodiode (Till Photonics). The dual-flash stimulus was programmed such that the first low-energy flash generates lower $[Ca^{2+}]_i$ to deplete the HCSP, followed by a second high-energy flash inducing higher $[Ca^{2+}]_i$ to release the LCSP.

To measure the $[Ca^{2+}]_i$ responses of LβT2 cells to GnRH, intact cells were loaded with Fura-2 AM (Molecular Probes) by immersing the cells in 2 μM Fura-2 AM containing bath solution for 30 min. $[Ca^{2+}]_i$ was then measured using the same dual wavelength excitation method as for the flash experiment.

Data analysis

Data analysis was performed in IGOR Pro 4.02 (WaveMetrics, Lake Oswego, OR), and results were presented as mean ± SEM with the indicated number of experiments. Statistical significance was evaluated using Kruskal–Wallis Analysis of Variance on Rank in SigmaStat 3.11 (Systat Software, Inc., CA) for multiple comparisons of groups with non-normal distribution. $P < 0.05$ was considered to be statistically significant. The off-line analysis of flash and ramp $[Ca^{2+}]_i$ data was performed as previously described (Sorensen et al., 2002; Yang et al., 2005). For ramp $[Ca^{2+}]_i$ experiments, we first differentiated the C_m trace to get the release rate. Then two components with different release rates were separated (see Fig. 2 bottom). The first component was regarded as fusion of vesicles from HCSP, and the second as from LCSP. The size and release course for each pool were calculated by integrating their fitted rate curves (Fig. 3A). The rate constant was determined as the rate divided by the remaining pool, which is calculated by subtraction of the released amount from the total pool size. The Ca^{2+} dependence of exocytosis is examined by plotting the rate constant versus $[Ca^{2+}]_i$ on a double logarithmic scale (Fig. 3C). For C_m responses to flash photolysis, the rate constants were obtained by fitting the exocytotic burst with double exponentials.

RESULTS

The elevation and oscillation of $[Ca^{2+}]_i$ in LβT2 cells induced by GnRH

Pituitary gonadotropes are characterized by their robust $[Ca^{2+}]_i$ oscillation in response to GnRH (Hille et al., 1994; Thomas and Waring, 1997), the physiological secretagogue for gonadotropes. To confirm that our LβT2 cells respond normally to GnRH, we challenged intact cells with 10 nM GnRH via local perfusion. As shown in Figure 1A, GnRH application produced robust $[Ca^{2+}]_i$ elevation superimposed with small amplitude oscillations. On average, GnRH significantly elevated the basal $[Ca^{2+}]_i$ from 150 ± 26 nM to a peak value of $1{,}100 \pm 57$ nM (Fig. 1B). The $[Ca^{2+}]_i$ response to GnRH is consistent with previous results in LβT2 cells albeit with higher $[Ca^{2+}]_i$ levels (Thomas et al., 1996), but significantly different from that of primary gonadotropes with much reduced $[Ca^{2+}]_i$ oscillation (Tse et al., 1993). This may reflect the subtle difference in the Ca^{2+} signaling between primary cultured cells and the corresponding immortal cell line.

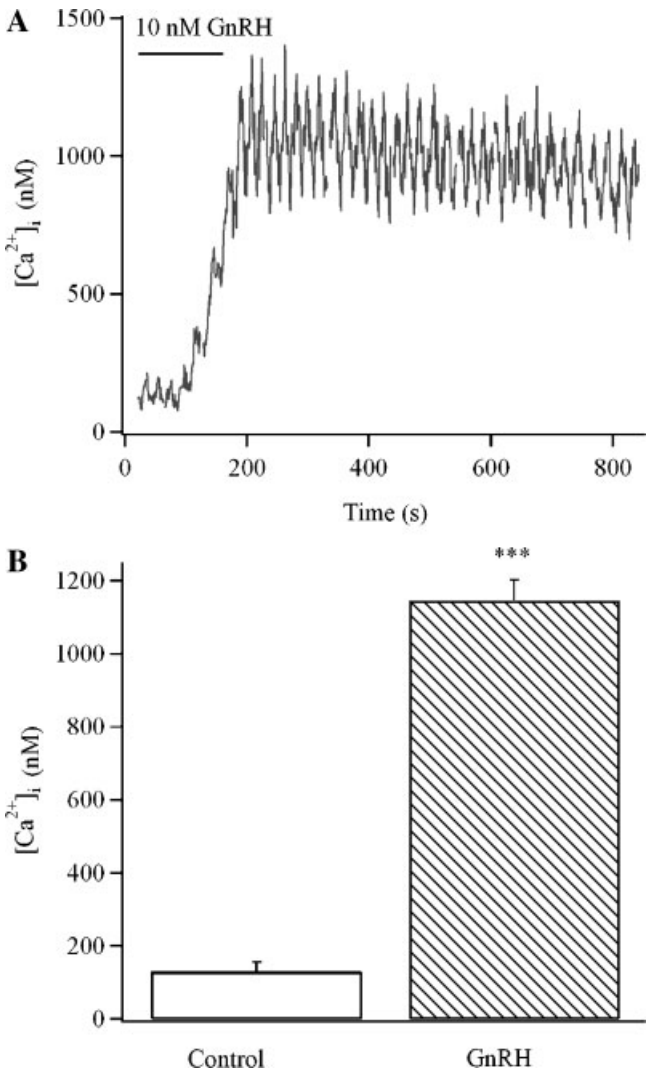

Fig. 1. LβT2 cell response to GnRH stimulation with $[Ca^{2+}]_i$ oscillation. **A**: Gobal $[Ca^{2+}]_i$ elevation and oscillation in LβT2 cell induced by 10 nM GnRH. An example trace showing that GnRH increases the global free $[Ca^{2+}]_i$ level and elicits periodical Ca^{2+} oscillations. $[Ca^{2+}]_i$ was measured by microfluoremetry in intact cells loaded with fura2-AM. Bar indicates the perfusion of GnRH (10 nM) through a glass pipette pointing at the cell. **B**: Summary of the $[Ca^{2+}]_i$ levels before and after application of GnRH. The $[Ca^{2+}]_i$ was increased from 150 ± 26 nM to a peak value of $1{,}100 \pm 57$ nM ($n = 12$, paired *t*-test, *** $P < 0.001$).

Distinct phases of Ca^{2+}-stimulated exocytosis in LβT2 cells

We next characterized the exocytosis stimulated by $[Ca^{2+}]_i$ elevation in LβT2. Exocytosis of a vesicle incorporates its vesicular membrane into the plasma membrane. This leads to an increase in the cell-surface membrane, which can be monitored electrically as an increase in C_m since it is proportional to the cell-surface membrane (Neher and Marty, 1982). C_m measurement has the advantage of monitoring secretion from the whole population of vesicles with millisecond time resolution. To be able to determine the release kinetics of different pools of vesicles, which depend steeply on $[Ca^{2+}]_i$, one has to resort to a "well-defined" $[Ca^{2+}]_i$ stimulation. Here we used Ca^{2+} uncaging from photolabile caged-Ca^{2+} to generate a special homogenous increase in $[Ca^{2+}]_i$, thus avoiding the complications of Ca^{2+} microdomains associated with Ca^{2+} influx and Ca^{2+} mobilization (Neher, 1998). In the first set of experiments, we generated a ramp increase in $[Ca^{2+}]_i$ via steady UV illumination. Under our experimental conditions, $[Ca^{2+}]_i$ could be gradually increased from a basal level to ~8 μM within seconds (Fig. 2), which acts as a titration of exocytosis with intracellular Ca^{2+}. The capacity to derive the entire Ca^{2+} sensitivity as well as Ca^{2+} cooperativity from a single ramp Ca^{2+} stimulation has the advantage of minimizing cell-to-cell variability. Secretion from LβT2 cells evoked by ramp Ca^{2+} exhibited two different secretory phases (Fig. 2, middle part, solid sigmoid trace): a small component (32 ± 4 fF, $n = 20$) with a low Ca^{2+} threshold (0.5 ± 0.06 μM) demonstrating a higher Ca^{2+} sensitivity; and a large one (172 ± 24 fF) which only started to release at a higher Ca^{2+} level (threshold $= 5 \pm 0.2$ μM). The Ca^{2+} threshold was defined as the $[Ca^{2+}]_i$ level at the half-maximal rate of exocytosis (Xu and Bajjalieh, 2001). The small size and very high Ca^{2+}-sensitivity of the first phase is reminiscent of the recently discovered HCSP in rat primary pancreatic β cells (Wan et al., 2004), chromaffin cells (Yang et al., 2002), and rat insulinoma INS-1 cells (Yang and Gillis, 2004). The second phase has a much larger size with a low Ca^{2+}-sensitivity. We have termed the two phases HCSP and LCSP here to reflect their differences in the Ca^{2+}-sensitivity.

Dependence of the kinetics of release on $[Ca^{2+}]_i$ for HCSP and LCSP

It has been recognized that neurotransmitter and hormone release require a highly cooperative Ca^{2+} stimulation. Direct measurement of the relationship between the rate of exocytosis and the concentration of Ca^{2+} near release sites in a variety of neuron and endocrine cells has revealed the third- or fourth-order cooperative nature of exocytosis (Heidelberger et al., 1994; Xu et al., 1998; Schneggenburger and Neher, 2000; Voets, 2000; Beutner et al., 2001). This has been taken as evidence that the final Ca^{2+} triggered fusion requires binding of at least 3–4 Ca^{2+} ions to the Ca^{2+} sensor(s). To further characterize the relationship between $[Ca^{2+}]_i$ and the release rate of the HCSP and LCSP, we tried to separate the two phases and derive their rates of release as previously introduced by Sorensen et al. (2002). As shown in Figure 3, the C_m in response to ramp $[Ca^{2+}]_i$ can be separated into two monophasic sigmoidal increases starting at different $[Ca^{2+}]_i$ thresholds. The two phases differ remarkably when we plotted their rate constants of release versus the $[Ca^{2+}]_i$ on a double logarithmic scale (Fig. 3C). HCSP has a much higher apparent affinity (indicated by the y-intercept) for Ca^{2+} than LCSP. Interestingly, the slope of the curves, which is indicative of the Ca^{2+} cooperativity of exocytosis, is 1.5 for HSCP and 2.7 for LCSP. The less Ca^{2+}-cooperative feature of the release from the HCSP is consistent with previous results in gonadotropes (Yang et al., 2005) and pancreatic β cells (Yang et al., 2002; Wan et al., 2004).

To validate our results from the ramp Ca^{2+} experiments and exclude the possibility that the slow down of the first phase of secretion was due to adaptation as proposed previously (Hsu and Jackson, 1996), we employed the technique of flash photolysis. Using flash uncaging, $[Ca^{2+}]_i$ can be elevated transiently to concentrations greater than 10 μM within 2–

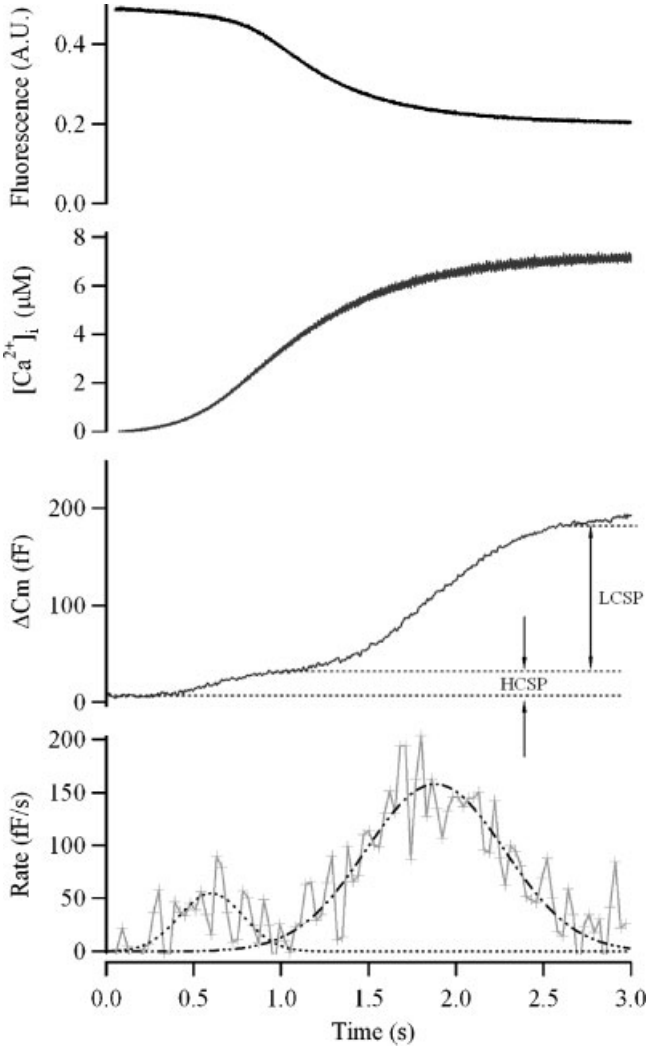

Fig. 2. Biphasic release induced by $[Ca^{2+}]_i$ ramp from LβT2 cells. $[Ca^{2+}]_i$ ramp was generated by the slow photorelease of Ca^{2+} from caged-Ca^{2+} via steady 380 nm UV-illumination. Traces displayed from top to bottom are fluorescence raw date of fura-6F (in arbitary unit), calculated $[Ca^{2+}]_i$, C_m response and derivate of the C_m curve, respectively. In response to the gradual $[Ca^{2+}]_i$ increase, the C_m response displays two distinct phases of release. The rate of release (bottom, noisy trace), which was derived by differentiating the C_m curve, could be best fitted with a sum of two distinct Gaussian functions indicated by two smooth curves (dashed lines). The $[Ca^{2+}]_i$ levels at the half-maximal rate of release of the two phases were taken as their respective $[Ca^{2+}]_i$ thresholds for fusion. The two phases may represent the exocytosis from two distinct pools of vesicles with substantially different Ca^{2+} sensitivities, and thus we termed the pools HCSP and LCSP. The $[Ca^{2+}]_i$ thresholds were 0.5 μM and 5 μM for HCSP and LCSP, respectively.

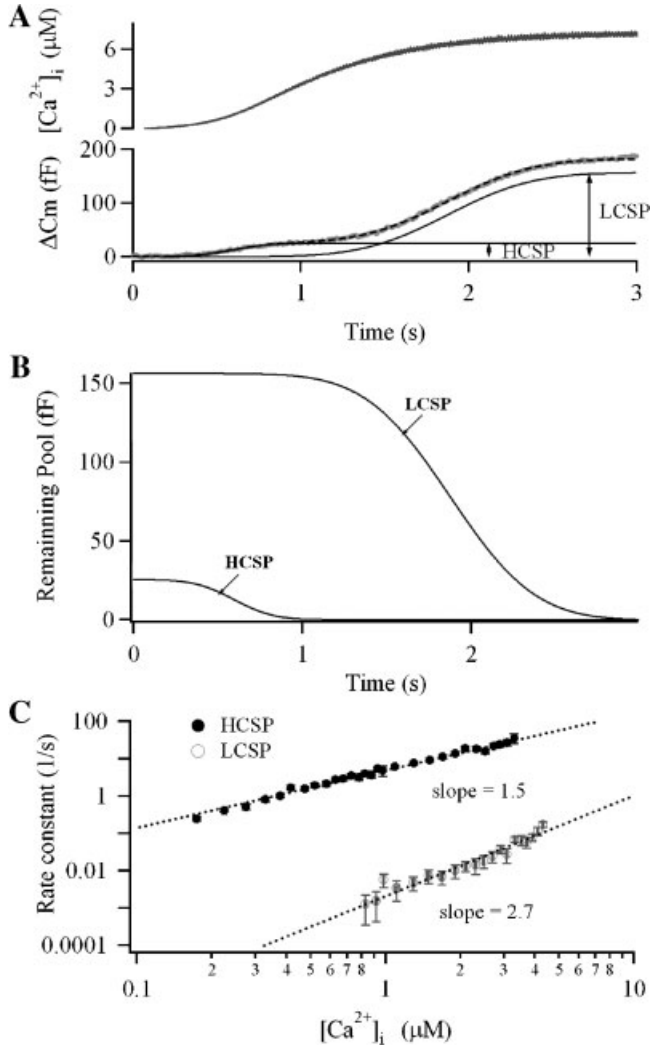

Fig. 3. HCSP and LCSP display different Ca^{2+} dependences. **A**: Separation of the two phases of secretion evoked by $[Ca^{2+}]_i$ ramp. By fitting the biphasic C_m curve with a sum (dashed line) of two integrations of Gaussian functions (smooth curves), we can separate the exocytotic responses into two distinct pools, HCSP and LCSP. **B**: The remaining pool sizes of HCSP and LCSP were calculated by subtraction the released amount from the total pool size. **C**: Calcium cooperativity of secretion for HCSP (gray solid circle, n = 12) and LCSP (open circle, n = 12). The rate constant was calculated as the release rate divided by the remaining pool size, and plotted versus $[Ca^{2+}]_i$ on a double logarithmic scale. The slopes of fitted traces (dashed lines) for HCSP and LCSP are indicated.

3 msec of the flash and remain relatively constant for several seconds during the measurement, thus mimicking a well defined step-like $[Ca^{2+}]_i$ elevation. This type of stimulus is necessary to assay the size and fusion kinetics of vesicle pools. As shown in Figure 4A, a $[Ca^{2+}]_i$ step to ~8 μM elicited an exocytotic burst component (within 1 sec after flash) followed by a linear sustained release (Fig. 4A). Detailed examination of the exocytotic burst revealed a double exponential feature (inset of Fig. 4A), indicating the presence of two releasable vesicle pools with distinct release kinetics. We fitted the overall C_m response with a triple exponential function. The exocytotic burst can be best described with a sum of two exponential functions: a small (30 fF) but very fast component with a rate constant ($1/\tau$) of 40 sec^{-1}, and a large slow component (150 fF) with a rate constant of 6 sec^{-1}. In Figure 4B, we compared the Ca^{2+} cooperativities of the fast and slow burst components, which presumably represent the fusion of the aforementioned HCSP and LCSP, by plotting their rate constants against the post-flash $[Ca^{2+}]_i$ levels on a double logarithmic scale. Again, it appears that the fast burst has a reduced Ca^{2+} cooperativity compared with that of the slow burst, agreeing with our ramp Ca^{2+} experiments.

If the HCSP and LCSP are distinct pools of vesicles differing in their sensitivities to Ca^{2+}, it should be possible to selectively deplete one pool while keeping the other. We thus devised a dual-flash protocol with different UV intensities. The first flash was adjusted to have a low intensity and triggered a small increase in $[Ca^{2+}]_i$. The second strong flash was given 4 sec apart to elevate $[Ca^{2+}]_i$ to a higher level. As shown in Figure 5, the first flash gave rise to an elevation of $[Ca^{2+}]_i$ to ~4.8 μM. In response to this $[Ca^{2+}]_i$ elevation, the C_m response can be fitted with a double exponential. There is a very fast burst component

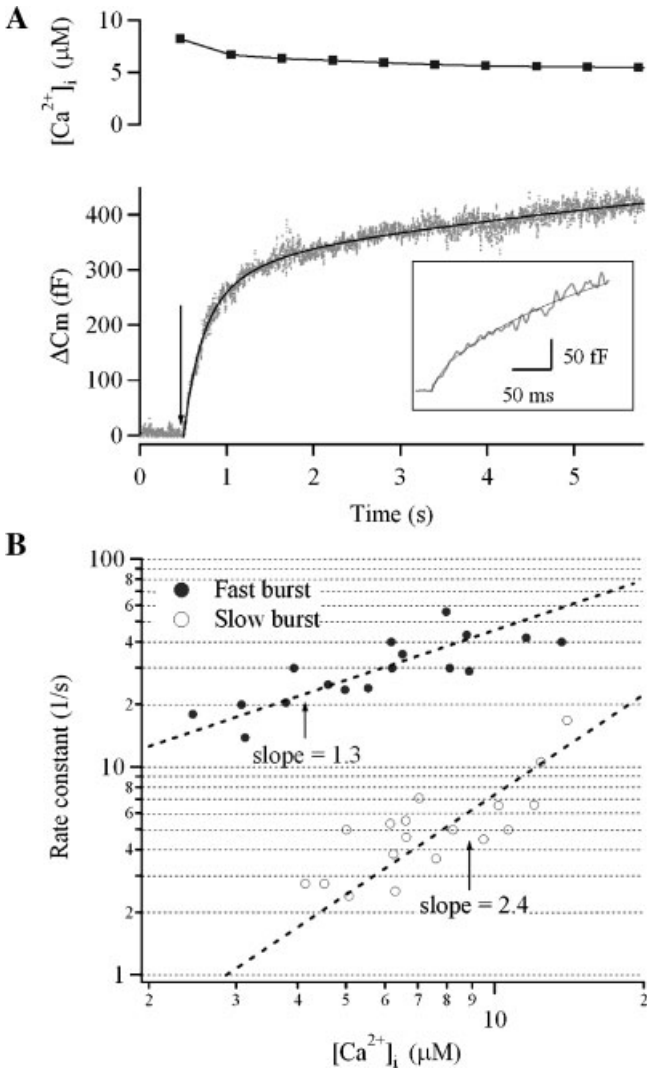

Fig. 4. Step-like $[Ca^{2+}]_i$ elevation elicits an exocytotic burst composed of two distinct components with different Ca^{2+}-dependences. **A**: Typical C_m response to step-like $[Ca^{2+}]_i$ elevation generated by flash photolysis. The C_m response can be best fitted with a sum of three exponential components (superimposed smooth line). The first two components are demonstrated at higher time resolution in the inset, which we call fast burst and slow burst component. The third component corresponds to the sustained release. Arrow indicates the onset of the flash. **B**: The fast and slow burst components display distinct Ca^{2+}-cooperativity. The rate constants from the double exponential fit to the exocytotic bursts were plotted against the post-flash $[Ca^{2+}]_i$. Superimposed broken lines are line fits for fast and slow component, respectively.

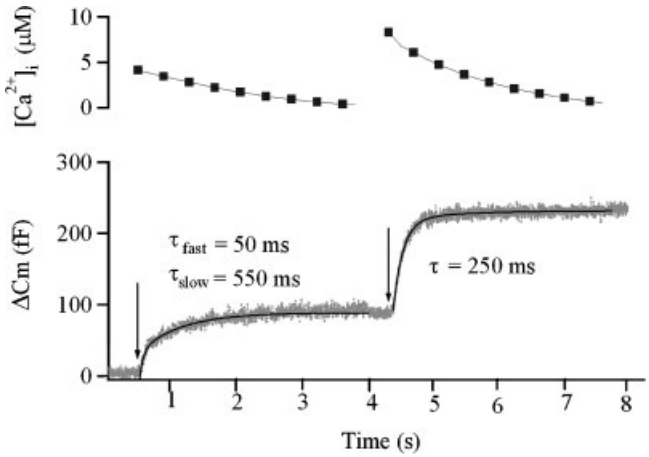

Fig. 5. Dual-flash stimulus confirms the existence of two pools of vesicles with different Ca^{2+} sensitivities. The cell was first stimulated with a weak UV flash (indicated by the first arrow), which gave rise to a small elevation of $[Ca^{2+}]_i$ to ~4.8 μM. The C_m response to this $[Ca^{2+}]_i$ elevation can be better fitted with double exponentials (superimposed solid curve). The time constants of the double exponentials are indicated. After the depletion of the fast burst component, subsequent elevation of $[Ca^{2+}]_i$ to about 8 μM by a stronger flash 4 sec later (indicated by the second arrow) triggered a single exponential response with a much larger amplitude (150 fF) and a time constant of 250 msec. The solid curve superimposed on the second response is a single exponential fit.

of exocytosis with an amplitude of 35 fF and a rate constant of 20 sec^{-1}, reflecting the fusion of HCSP. After the depletion of the HCSP, subsequent elevation of $[Ca^{2+}]_i$ to about 8 μM triggered a much larger component (150 fF) of release with a single rate constant of 4 sec^{-1}, corresponding to the remaining LCSP with a much lower affinity to Ca^{2+}. It should be noted that part of the LCSP will also be released during the first flash, as indicated by the second slow component with a rate constant of 1.8 sec^{-1}. A similar response was observed in all six cells examined with similar dual-flash stimulation.

Differential modulation of the HCSP and LCSP

Protein kinases are important modulators of the amplitude of exocytosis as well as its Ca^{2+}-sensitivity (Gillis et al., 1996a; Smith et al., 1998; Smith, 1999; Wu and Wu, 2001; Yang et al., 2002, 2005; Zhu et al., 2002; Wan et al., 2004; Yang and Gillis, 2004; Lou et al., 2005). We thus tested the effects of PKC, PKA, or GnRH on the size of HCSP and LCSP in LβT2 cells. We activated PKC and PKA by PMA and forskolin, respectively. As exemplified in Figure 6A, PMA treatment (100 nM) significantly increased the amplitude of HCSP without affecting the size of LCSP. Forskolin (10 μM), on the other hand, exerted no obvious effect on either HCSP or LCSP (Fig. 6B). Interestingly, GnRH (10 nM), which also activates PKC and PKA through its G protein-coupled receptor (Garrel et al., 1997; Liu et al., 2002; Millar et al., 2004), dramatically augmented the size of both HCSP and LCSP (Fig. 6C). Figure 7 summarizes the effects of PMA, Forskolin, GnRH, on HCSP and LCSP in comparison to the control. The amplitude of the HCSP in PMA-treated cells (77 ± 10 fF, $n = 24$) is about 2.5-fold higher than in control cells (31 ± 3 fF, $n = 20$). The amplitude of the LCSP does not differ significantly between PMA-treated cells (158 ± 11 fF) and control cells (170 ± 13 fF). The stimulatory effect of PMA was blocked by a specific PKC antagonist, Gö6976 (500 nM), demonstrating the involvement of PKC activation. For forskolin-treated cells ($n = 15$), the amplitudes of HCSP and LCSP are 32 ± 4 fF and 157 ± 22 fF, respectively, which were not significantly different from those of control cells.

Interestingly, GnRH showed the most dramatic effects on the pool size of both HCSP and LCSP. As shown in Figure 7, GnRH treatment for 2–3 min increased the pool size to 110 ± 18 fF ($n = 18$) and 248 ± 15 fF ($n = 18$) for HCSP and LCSP, respectively. The stimulatory effects of GnRH were only partially inhibited by Gö6976, suggesting the involvement of another signal other than PKC. GnRH signaling involves not only PKC activation, but also IP_3 production and intracellular Ca^{2+} mobilization from IP_3-sensitive Ca^{2+} stores (Tse et al., 1997; Kraus et al., 2001; McArdle et al., 2002; Krsmanovic et al., 2003; Ruf et al., 2003). We tested whether IP_3-sensitive Ca^{2+} release could participate in mediating the residual effects of GnRH after PKC inhibition. Indeed, pretreatment with both Gö6976 and heparin (an IP_3 receptor inhibitor, 1 mg/ml by intracellular perfusion) for 2–3 min completely abolished the stimulatory effects of GnRH on the size of HCSP and LCSP. This result suggests that, in addition to PKC, Ca^{2+} plays an important role in regulating the pool size, in agreement with the previous findings in chromaffin cells (von Ruden and Neher, 1993; Smith et al., 1998). Consistence with this hypothesis, we found that elevating the basal $[Ca^{2+}]_i$ from

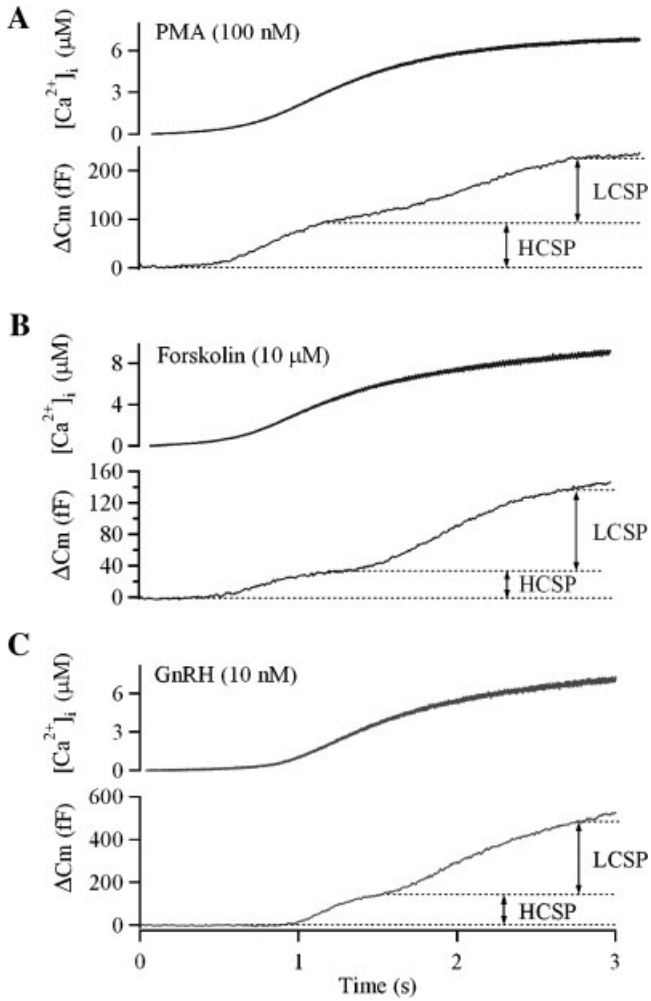

Fig. 6. Example C_m responses to ramp $[Ca^{2+}]_i$ from cells treated with either PMA, forskolin, or GnRH. **A**: The cell was pretreated with 100 nM PMA for 2–3 min before the $[Ca^{2+}]_i$ ramp. It displays a larger HCSP followed by a normal size of LCSP as compared with control cells, indicating PMA preferentially augments the size of HCSP. **B**: Forskolin (10 μM with 2–3 min pre-treatment) exerted no observable effect on the size of both HCSP and LCSP. **C**: Pre-treatment with 10 nM GnRH for 2–3 min dramatically increased both HCSP and LCSP. Please note the difference in scale of ΔCm between (C) and (A–B).

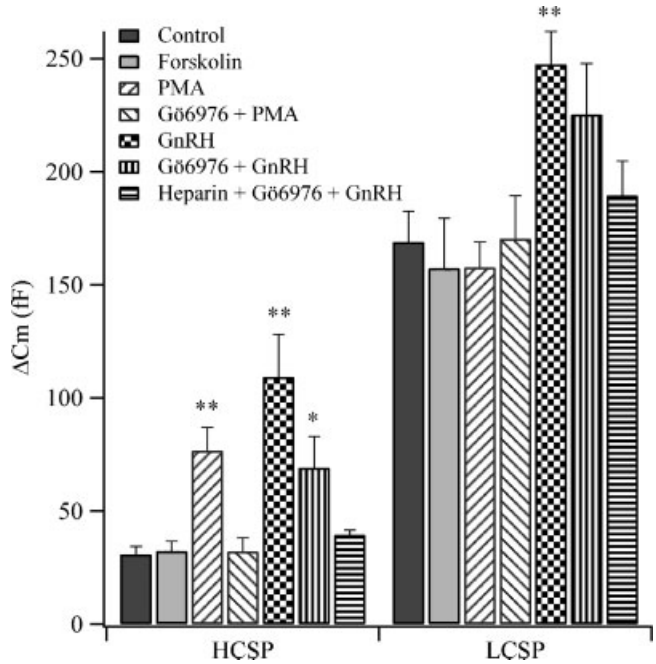

Fig. 7. Summary of the effects of various treatments on the size of HCSP and LCSP in LβT2 cells. All data are displayed as mean ± SEM summarized from more than 15 cells. * and ** denote significant difference $P < 0.05$ and $P < 0.01$ compared with control cells, respectively.

150 nM to 330 nM increased the pool size of HCSP from 31 ± 3 fF (n = 26) to 140 ± 9 fF (n = 25) in LβT2 cells.

DISCUSSION

In the present study, we have identified two distinct phases of secretion, which we have called HCSP and LCSP here. The HCSP has a very high Ca^{2+} sensitivity (Ca^{2+} threshold ~0.5 μM) with a low Ca^{2+} cooperativity, whereas the LCSP has a much lower sensitivity (Ca^{2+} threshold ~5 μM) with a steeper Ca^{2+} cooperativity. It is of interests to compare our HCSP and LCSP with various pools of vesicles found in other cells. In chromaffin cells, release ready vesicles are composed of two sequentially arranged pools: the RRP and SRP (Voets et al., 1999). The HCSP found in this study (rate constant of 10–20 sec^{-1} at 3 μM) has much faster release kinetics than for the RRP (rate constant of 1 sec^{-1} at 3 μM) (Voets et al., 1999), whereas the release kinetics of the LCSP (rate constant of 1–2 sec^{-1} at 3 μM) are comparable with those of the RRP. Although a small HCSP has also been shown in chromaffin cells and pancreatic β cells in addition to the previous well-characterized RRP (Yang et al., 2002; Wan et al., 2004; Yang and Gillis, 2004), it is hard to separate the HCSP from the RRP in those cells. In contrast, the HCSP in LβT2 cells can be clearly distinguished from the LCSP. A similar co-existence of vesicle populations with remarkable differences in Ca^{2+} affinity has been documented in human neutrophils (Nusse et al., 1998). It is not clear whether the HCSP and LCSP represent the same type of vesicles arranged sequentially or they represent different types of vesicles with different Ca^{2+}-sensing machineries. Further identification of the molecular markers of these two pools will be necessary to answer this question.

Recent studies have suggested that the Ca^{2+}-sensing process of membrane fusion can be modulated in a wide spectrum of cells (Yang et al., 2002, 2005; Zhu et al., 2002; Wan et al., 2004; Yang and Gillis, 2004; Lou et al., 2005). In the present study, application of PMA but not forkolin increases the size of HCSP by about threefold without significantly affecting the LCSP size. Interestingly, GnRH produces a much larger augmentary effect on both HCSP and LCSP than that of PMA. GnRH not only activates PKC but also stimulates intracellular Ca^{2+} release via IP_3-sensitive Ca^{2+} stores (Tse et al., 1997; Kraus et al., 2001; McArdle et al., 2002; Krsmanovic et al., 2003; Ruf et al., 2003). We found that PKC blockade by Gö6976, while completely abolishing the effect of PMA, only partially inhibited the effect of GnRH. Combination treatment with Gö6976 and heparin, an inhibitor of the IP_3 receptor, completely abolished the effect of GnRH. Furthermore, we showed that the pool size of HCSP is sensitive to basal $[Ca^{2+}]_i$. Taken together, we conclude that HCSP is regulated by both PKC and Ca^{2+} in an additive manner. The mechanism by which PKC and Ca^{2+} augment the size of HSCP remains to be elucidated. It is possible that PKC and Ca^{2+} accelerate the maturation of vesicles to the HCSP. Indeed, PKC activation and Ca^{2+} has been shown to enhance the recruitment of vesicles to the RRP (Gillis et al., 1996b; Smith et al., 1998; Smith, 1999; Nagy et al., 2002). It is of interest to note that Munc13 proteins (Ashery et al., 2000; Betz et al., 2001), which are essential regulators of vesicle priming, possess calmodulin-binding sites that can be activated by Ca^{2+} (Junge et al., 2004). It has also been demonstrated that F-actin network acts as a barrier to the secretory vesicles blocking their access to exocytotic sites. PKC and Ca^{2+} have been proposed to increase the transport of vesicles from the reserve compartment to the release-ready vesicle compartment via remodeling of cortical

F-actin (Vitale et al., 1995; Trifaro et al., 2000). Gonadotropes are stimulated to produce pulsatile secretion in response to intracellular Ca^{2+} released from Ca^{2+} stores (Thomas and Waring, 1997; Tse et al., 1997). Hence, GnRH-induced Ca^{2+} mobilization not only plays a triggering role in secretion, but also acts as an important modulator for the size of the releasable vesicles. The shifting of more vesicles to a highly Ca^{2+}-sensitive status by GnRH signaling would dramatically increase the secretory responsiveness to $[Ca^{2+}]_i$. This mechanism may thus play an important role in the stimulus-secretion coupling of gonadotropin secretion.

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