Ca\(^{2+}\)-dependent Exocytotic Pathways in Chinese Hamster Ovary Fibroblasts Revealed by a Caged-Ca\(^{2+}\) Compound*

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Ca\(^{2+}\)-dependent exocytosis and endocytosis of Chinese hamster ovary (CHO) fibroblasts were investigated using capacitance measurement and rapid photolysis of a caged-Ca\(^{2+}\) compound, dimethoxynitrophenamine tetrasodium salt. CHO cells exhibited large and fast increases in membrane capacitance (1.9 ± 1 picofarads, or 13 ± 7% of total membrane area, mean ± S.D., n = 37) upon Ca\(^{2+}\) jumps to [Ca\(^{2+}\)], larger than 20 μM. The fast exocytosis occurred with a delay (20–80 ms), and exhibited a rate constant that was strongly dependent on [Ca\(^{2+}\)]. The maximal rate constant of exocytosis was 2.8/s, and a half-maximal rate was achieved at 30 μM. The fast exocytosis was followed by rapid endocytosis in 28% of the cells. The endocytosis often began after a delay of 0.5–2 s. Ca\(^{2+}\) jumps also induced stepwise increases in membrane capacitance of 10–134 femtofarads in 40% of the cells, indicating fusion of large vesicles with diameters of 0.4–1.5 μm. The exocytosis of the large vesicles could selectively be induced with smaller Ca\(^{2+}\) jumps (6–20 μM), and occurred slowly with a rate constant of 0.2/s. These data indicate that CHO fibroblasts possess Ca\(^{2+}\)-dependent exocytotic mechanisms. Moreover, two parallel exocytotic pathways may exist reminiscent of those of neurons and endocrine cells. A kinetic model was constructed to account for the fast exocytosis of CHO cells.

Ca\(^{2+}\)-dependent exocytotic secretion of neurotransmitters and hormones has been considered as a function representative of neurons and endocrine cells (1–3). It is well established that neurons possess two distinct types of secretory vesicles, the synaptic vesicles carrying classical neurotransmitters and large dense-core vesicles carrying neuropeptides. Some endocrine cells also possess synaptic-like microvesicles in addition to dense-core vesicles (4). The synaptic-like microvesicles are generated by recycling between the plasma membrane and early endosomes as in the case of the synaptic vesicles (1), and they express synaptophysin as a common marker. Recently, it has been reported that even non-secretory cells possess recycling membrane pathways, and transfected synaptophysin selectively distributes to these early endosomal vesicles (5, 6). In addition, putative Ca\(^{2+}\) sensor molecules of exocytosis, synaptotagmins, are ubiquitously expressed (7). Furthermore, Ca\(^{2+}\)-dependent secretion of a fast neurotransmitter, ACh, has been reported in muscles and fibroblasts that were incubated with ACh (8, 9). In order to characterize Ca\(^{2+}\) and time dependence of exocytosis of non-secretory cells, we investigated exocytosis of CHO fibroblasts using time-resolved capacitance measurement in combination with rapid photolysis of a caged-Ca\(^{2+}\) compound (10–12). We found that the CHO cells undergo massive increases in membrane capacitance upon large increases in [Ca\(^{2+}\)], and exhibited several characteristics similar to those found in one type of nerve terminal and endocrine cells (10–13). The capacitance increases occurred with a rate constant of 2.8/s and exhibited low sensitivity to Ca\(^{2+}\). Some cells even showed rapid endocytosis as in endocrine cells. Interestingly, we detected stepwise capacitance increases of 10 fF or larger in 40% of the cells, suggesting fusion of vesicles with diameters of 0.4 μm or greater. The latter form of exocytosis occurred slowly and could be selectively induced by a small increase in [Ca\(^{2+}\)] (≈6 μM). Thus, the fibroblasts have been found to possess two exocytotic pathways, which might correspond to the small-dense and large dense-core vesicles in neurons and endocrine cells.

EXPERIMENTAL PROCEDURES

Preparation of Cells—CHO cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Life Technologies, Inc.) in an atmosphere of 10% CO\(_2\) at 37 °C. They were passaged approximately once a week, and plated on 5-mm cover glasses (No. 0, Menzel Glass, Braunschweig, Germany) in 96-well culture plates 1 to 3 days before the patch clamp experiments.

Recording Solutions—The external recording solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM Na-HEPES, and 20 μM glucose at pH 7.4 and 320 mosm. A caged-Ca\(^{2+}\) compound and other chemicals were dissolved in a basic internal solution containing 120 mM Cs-glutamate, 10 mM CsCl, 40 mM Cs-HEPES, and 200 μM benzothiazole coumarin (Molecular Probes, Eugene, OR) at pH 7.3. As a caged-Ca\(^{2+}\) compound, we used 10 mM DM-nitrophen (dimethoxytrinitrophenamine tetrasodium salt; Calbiochem, La Jolla, CA) loaded with 4 mM CaCl\(_2\). The osmolarity of the internal solutions was about 320 mosm after addition of these chemicals.

Intermediate[Ca\(^{2+}\)] levels were created by loading DM-nitrophen with lesser amounts of CaCl\(_2\) (2.5, 2, 1 or 0.5 mM). We carried out all experimental procedures under yellow illumination (FL40S-Y-F, National, Tokyo) at room temperature, 22–25 °C.

Capacitance Measurements—The capacitance measurements were carried out using a patch clamp amplifier, AxoPatch 1D (Axon Instruments, Foster City, CA), and the phase tracking method (12, 14). A computer-based lock-in amplifier was constructed using the C programming language and a personal computer (NEC 9821An, Tokyo, Japan) (15). The cells were held at −20 mV, at which current-to-voltage relationships became almost linear soon after the whole cell recording and to which 1 kHz sine waves with a peak to peak amplitude of 100 mV were applied. Capacitance and conductance were calculated from 10 cycles of sine waves and stored at 83 Hz, or they were calculated from one cycle of sine wave and stored at 1 kHz for the experiments using a

*This work was supported by grants-in-aid from the Japanese Ministry of Education, Science, and Cultures, a research grant from the Human Frontier Science Organization, and a grant from the Takeda Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: [Ca\(^{2+}\)], intracellular Ca\(^{2+}\); CHO, Chinese hamster ovary; DM-nitrophen, dimethoxytrinitrophenamine tetrasodium salt; APTRA, 2-aminothiazole-3-N,O-triacetic acid; F, farad(s).
flask lamp. The phase tracking was performed every 7 s. The Ca\(^{2+}\) jumps often induce transient inward currents (10). In order to minimize the error in the estimates of membrane capacitance due to the current, we selected our data according to the following criteria: 1) resistance of seal before photolysis was larger than 1 GΩ; 2) access resistance was smaller than 8 MΩ; and 3) amplitude of the transient current was less than 5 pA. Omitting by these criteria, the maximal error in the estimate of capacitance should be smaller than 0.16% of total membrane capacitance (16). Our studies were based on a total of 77 cells meeting these criteria. Whole cell membrane capacitance ranged between 8 and 30 pF (mean ± S.D. = 14.7 ± 6.7 pF).

**Ca\(^{2+}\)** Measurements—Measurement of [Ca\(^{2+}\)] was performed using a ratiometric long wavelength Ca\(^{2+}\)-indicator dye, benzothiazole coumarin (17). Two monochromatic lights with wavelengths of 430 and 480 nm were isolated from a xenon lamp, rapidly alternated using a monochromator (T.I.L.L. Photonics, Munich, Germany) and fed into one port of a light guide (IX-RFA-caged, Olympus). The excitation light was reflected by a dichroic mirror, DM500, placed beneath the objective, and fluorescence light emitted from the cells was further filtered with an LP520 (Melles Griot, Stanford, CA). The detection mode was in the line scanning mode of a confocal microscope (MRC600, Bio-Rad) (21). The activation of the caged compounds was estimated as 12 ms using the time constant of the fluorescence ratio changes. The time constant of the fast exocytosis was calculated from the peak fluorescence ratio change (Fig. 1 F).

**Compounds—** A photolysed form of caged Ca\(^{2+}\)-indicator dye, benzothiazole coumarin was utilized to estimate the peak values of [Ca\(^{2+}\)] in CHO cells (19). We prepared solutions containing 20 mM EGTA, 20 mM Ca\(^{2+}\) (Fig. 1 A), and a droplet containing 200 mM fluo-3, 100 mM Ca\(^{2+}\) (Fig. 1 B), and 50 mM Ca\(^{2+}\) (Fig. 1 C). The speed of photolysis should not much affect the rate of the fast exocytosis, since the maximal rate constant of the fast exocytosis was below 3 s\(^{-1}\). The kinetics of the fast exocytosis was not affected by the speed of the xenon flash lamp as a source of actinic light (n = 9), with which the photolysis could be carried out within a fraction of a millisecond (13).

**RESULTS**

The fast Ca\(^{2+}\)-dependent Exocytosis—Most CHO cells exhibited large increases in membrane capacitance when concentration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) were rapidly raised to more than 20 μM (Fig. 1, A, B, and D). The time course of the capacitance increase could be well described by a single exponential function with a time constant of 0.4–2 s (Fig. 1 B).

**Fig. 1.** Ca\(^{2+}\) and time dependence of the capacitance increases in CHO cells evoked by rapid Ca\(^{2+}\) jumps induced by photolysis of a caged-Ca\(^{2+}\) compound, DM-nitrophen. A and B, capacitance traces recorded from two cells. Ca\(^{2+}\) jumps reached 15 μM in A, 50 μM in B, C, a capacitance trace recorded from a cell where a xenon flash lamp was utilized to raise [Ca\(^{2+}\)]\(_{i}\) in 1 ms. An arrowhead indicates the onset of the capacitance rise. [Ca\(^{2+}\)]\(_{i}\) was measured 0.1 s before and 1 s after the flash photolysis. D, amplitudes of capacitance increase plotted against peak [Ca\(^{2+}\)]. Amplitudes were expressed as % of the total membrane area. Those cells that showed pure stepwise capacitance increases were excluded from this histogram. E, rate constants of capacitance increases plotted against [Ca\(^{2+}\)]. A dashed line represents a Hill equation with a Hill coefficient of 3.5. Each point in D and E represents a mean value for more than 6 cells. Horizontal bars show S.D. and vertical bars show S.E. F, delays in exocytosis measured in 9 cells. Solid lines in E and F represent predicted values estimated from the Equation 1 in the text.
The slow Ca\(^{2+}\)-dependent exocytosis was followed by decreases in the membrane capacitance in 28% of the cells (Fig. 2; 14 out of 49 cells). The amplitudes of the capacitance decreases evoked by Ca\(^{2+}\) jumps were 50-100 fF. Most capacitance changes occurred in a stepwise manner. The stepwise increases were followed by capacitance decreases, possibly reflecting endocytosis, which also occurred with a delay (Fig. 2). Unlike in the case of the large dense-core vesicle secretion in endocrine cells (22, 23), the endocytosis was never accompanied by a stepwise decrease in capacitance.

The slow Ca\(^{2+}\)-dependent exocytosis—Some large stepwise capacitance increases were detected in 40% of the cells where Ca\(^{2+}\) jumps larger than 5 \(\mu M\) were observed (Fig. 1C and Fig. 3B). The sizes of the steps ranged between 5 and 134 fF, representing the exocytosis of vesicles with diameters between 0.4 and 1.5 \(\mu m\). Notably, we found that the stepwise capacitance increases were selectively induced at low [Ca\(^{2+}\)]\(_{i}\) (<20 \(\mu M\)) where little fast exocytosis was detected (n = 9, Fig. 3A), indicating that this component of exocytosis has a higher affinity for Ca\(^{2+}\) than the fast exocytosis. The minimum [Ca\(^{2+}\)] at which the stepwise increases were observed was 6 \(\mu M\). Precise Ca\(^{2+}\) dependence of the stepwise exocytosis, however, was difficult to confirm, because the steps occurred infrequently. The numbers of steps (>10 fF) induced by a Ca\(^{2+}\) jump ranged between 1 and 10 (mean ± S.D. = 3.6 ± 2.6, n = 24).

The exocytosis of the stepwise capacitance increase was slower than the fast exocytosis. We estimated the speed of the stepwise exocytosis in those cells in which Ca\(^{2+}\) jumps larger than 30 \(\mu M\) were evoked. The cumulative latency histogram of the largest steps demonstrated that the rate constant of the slow exocytosis was about 0.3/s, about one-tenth that of the fast exocytosis.

**DISCUSSION**

We have found that the CHO fibroblasts display massive capacitance increases upon Ca\(^{2+}\) jumps, and identified two types of capacitance changes. The fast component of capacitance changes occurred smoothly and showed a lower affinity for Ca\(^{2+}\). In contrast, the slow capacitance increases occurred in a stepwise manner and exhibited a higher affinity for Ca\(^{2+}\). We will discuss below the possibility that these two types of capacitance changes reflect secretion via two distinct exocytotic pathways.

The fast components of capacitance changes of CHO cells showed many properties in common with the exocytosis reported in nerve terminals and endocrine cells. First, the capacitance changes exhibited an exponential time course and occurred with a delay (Fig. 1, B and C) (10, 12, 13, 24). Second, the capacitance changes could be exhausted by large Ca\(^{2+}\) jumps (Fig. 2B) (11, 23). Third, the capacitance changes showed a rather low sensitivity to Ca\(^{2+}\), and depend on Ca\(^{2+}\) in a cooperative manner (Fig. 1E) (11–13). Finally, the capacitance increases were followed by capacitance decreases, possibly reflecting endocytosis, which also occurred with a delay (Fig. 2) (12, 23, 24). Thus, capacitance changes detected in CHO cells were phenomenologically similar to those of secretory cells. The rate constant of the fast exocytosis in CHO cells, however, was one-tenth that of endocrine cells and one-thousandth of the neurons. These data were consistent with the hypothesis that CHO cells possess a Ca\(^{2+}\)-dependent exocytosis pathway akin to those in secretory cells, although its rate constant was far smaller. It can be speculated that the fast exocytosis of CHO cells more likely reflects exocytosis of the small synaptic-like microvesicles, because the endocytosis following the fast exocytosis was not associated with large stepwise decreases in the membrane capacitance, reflecting vacuolation, as in the case of secretion via the dense-core vesicles in endocrine cells (12, 22, 23).

In order to compare the kinetic features of the Ca\(^{2+}\)-dependent exocytosis in CHO cells and those in neuron, their rate
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We have found that CHO cells displayed stepwise increases in membrane capacitance, indicating the secretion of large secretory vesicles with diameters of between 0.4 and 1.4 μm. The large vesicle secretion was slower and showed a higher affinity to Ca\textsuperscript{2+} than the fast exocytosis (Fig. 3). Thus, the slow exocytosis in CHO cells is similar to the exocytosis of the dense-core vesicles in nerve terminals in that it was slower, more sensitive to Ca\textsuperscript{2+}, and mediated by larger secretory vesicles than the fast exocytosis (2, 12). The large vesicle exocytosis in CHO cells is, however, very infrequent compared with that of endocrine cells; none was detected in 60% of the cells, and, on average, 2.5 such exocytoses were detected.

Finally, the reason why the cell line possesses an abundance of Ca\textsuperscript{2+}-dependent exocytotic pathways should be considered. If such pathways were not necessary for survival or proliferation of the cell line, they might not have been evolutionarily preserved. They may traffic membrane proteins or lipids to the plasma membrane or secrete extracellular matrix proteins. Ca\textsuperscript{2+}-dependent incorporation of a membrane integral protein, NCAM, has been reported in neurons and endocrine cells (25). The Ca\textsuperscript{2+}-dependent exocytotic pathways might function also as constitutive secretory pathways, and not necessarily be coupled with Ca\textsuperscript{2+} rises, as in the case of secretion via synaptic vesicles. In fact, spontaneous secretion of ACh was detected in CHO cells (9). Electron microscopic identification of secretory vesicles responsible for the two components of capacitance changes in CHO cells would facilitate understanding of Ca\textsuperscript{2+}-regulated exocytosis.

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