Calcium dependence of exocytosis in lacrimal gland acinar cells

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Sundermeier, Thomas, Gary Matthews, Peter R. Brink, and Benjamin Walcott. Calcium dependence of exocytosis in lacrimal gland acinar cells. Am J Physiol Cell Physiol 282: C360–C365, 2002. First published October 10, 2001; 10.1152/ajpcell.00298.2001.—Simultaneous measurements of membrane capacitance and intracellular calcium concentration were used to examine the calcium dependence of exocytosis in single acinar cells from mouse lacrimal gland and to establish the quantitative relation between calcium concentration and rate of exocytosis. Application of adrenergic or muscarinic agonists elevated intracellular calcium and evoked exocytosis, as indicated by an increase in membrane capacitance of single cells. The capacitance response to agonist stimulation was eliminated by internal dialysis with the calcium buffer EGTA, which demonstrated that the increase in intracellular calcium was necessary for agonist-evoked exocytosis. When internal calcium was elevated by application of the calcium ionophore ionomycin, exocytosis was evoked in the absence of agonist stimulation. Thus an increase in intracellular calcium was necessary and sufficient for exocytosis in single acinar cells. The rate of change of membrane capacitance increased as approximately the third power of the calcium concentration, which is similar to the dependence of exocytosis rate on calcium concentration in other secretory cells.

The acinar cells of lacrimal glands secrete protein and water in response to both cholinergic and adrenergic stimulation (3, 14, 20). Muscarinic M3 receptors are coupled via G proteins, specifically Go11 and Gq (19), to phospholipase C, which ultimately results in an increase in intracellular calcium (7, 22). Similarly, activation of α-adrenergic receptors also increases intracellular calcium via G proteins (18), albeit via a different second messenger pathway (14) that to date has not been well defined. It is also well documented that activation of both M3 and α-adrenergic receptors induces secretion of protein from lacrimal gland tissue fragments (3, 20, 22, 24). Thus it has always been assumed that the increase in intracellular calcium induced by the agonists triggers the protein secretion. Such a linkage has been clearly demonstrated in endocrine cells (13) and at synapses (1, 10), where hormone and neurotransmitter release has been demonstrated to be directly affected by the level of intracellular free calcium. Such a relationship has not been directly demonstrated in lacrimal gland acinar cells, and the quantitative relationship has not been determined between the concentration of intracellular free calcium and the rate of exocytosis. Therefore, we have used combined patch-clamp and fura 2 measurements in cells isolated from mouse lacrimal glands and have simultaneously measured the membrane capacitance while manipulating the intracellular free calcium concentration ([Ca2+]i) to directly determine the causal relationship.

Capacitance measurements provide an index of cell surface area. As protein is secreted by exocytosis, vesicles fuse with the plasma membrane and increase its area, which is seen as an increase in capacitance. We then correlated those changes with the levels of free calcium in the same cell at the same time. We have shown that agonist-induced increases in free intracellular calcium caused significant changes in membrane capacitance. Introducing the calcium buffer EGTA into the cell blocked both the calcium and capacitance increase in response to agonist stimulation. Treating the cells with ionomycin, which elevated intracellular free calcium in the absence of receptor agonists, increased the membrane capacitance. Analysis of the data showed that the rate of exocytosis is proportional to the third power of [Ca2+]i.

MATERIALS AND METHODS

Cell isolation. For patch-clamp analysis, acinar cells were isolated from freshly dissected glands by a method developed for rat lacrimal glands (9). The mice were anesthetized with halothane and then decapitated. The glands were removed, placed in soy bean trypsin inhibitor (STI; 0.1 mg/ml; Sigma), and cut into small pieces with two sterile scalpel blades. The pieces were then washed with Hanks’ balanced salt solution and incubated at 37°C for 15 min. After being washed again with STI, the cells were incubated at 37°C in an agitated mixture of collagenase (225 U/ml; GIBCO), DNase (10 U/ml;...
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RESULTS

Capacitance changes stimulated by cholinergic and adrenergic agonists in lacrimal gland acinar cells. Neural inputs to the lacrimal gland stimulate protein exocytosis into the tear fluid by activating both cholinergic and adrenergic receptors (4, 26). Activation of both receptor types also releases calcium from internal stores via independent second messenger pathways (14). To establish the linkage between agonist-activated exocytosis and the concomitant changes in \([Ca^{2+}]_i\), in single acinar cells from the lacrimal gland, we combined fluorescence calcium imaging with time-resolved measurement of membrane capacitance as an index of exocytosis. As illustrated in Fig. 1, brief application of cholinergic agonists carbachol (CCh; 10 \(\mu M\)) increased intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) as illustrated in Fig. 1, brief application of carbachol (CCh; 10 \(\mu M\)) increased intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) as illustrated in Fig. 1, brief application of carbachol (CCh; 10 \(\mu M\)) increased intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)). Measurement of ([Ca\(^{2+}\)]\(_i\)), from fura 2 fluorescence was performed as detailed previously (11). Spatially averaged fluorescence measurements were made with a photomultiplier tube with a pinhole oriented to view the entire cell. Alternating excitation light at 360 and 390 nm was provided by a rotating filter wheel in the epifluorescence path of the inverted microscope. ([Ca\(^{2+}\)]\(_i\)) was calculated from the ratio of emitted light at the two excitation wavelengths with calibration constants obtained by dialyzing cells with solutions containing known, highly buffered concentrations of free calcium (11). For studies of the calcium dependence of the rate of exocytosis, only calcium levels up to 1,000 nM were analyzed. 

Electrophysiological recording. Isolated mouse lacrimal gland acinar cells were placed in an oxygenated external solution containing (in mM) 115 NaCl, 2.5 KCl, 1.0 CaCl\(_2\), 1.0 MgCl\(_2\), 10 HEPES, and 20 glucose (pH = 7.4). Patch pipettes were filled with an internal solution containing (in mM) 110 KCl, 1 MgCl\(_2\), 0.1 CaCl\(_2\), 1 N-methyl-D-glucamine (NMDG)-EGTA, 10 HEPES, and 0.11 mm 2-tetrapotassium salt (Molecular Probes, Eugene, OR) for fluorescence measurements of ([Ca\(^{2+}\)]\(_i\)). The solution pH was adjusted to 7.2. In some experiments, NMDG-EGTA concentration was increased to 10 or 20 mM. Whole cell voltage-clamp records were obtained with an EPC-9 patch-clamp amplifier under control of Pulse software (HEKA, Darmstadt, Germany). Seal resistances were >1 GΩ. After breaking in to begin whole cell recording, exchange of patch pipette and cellular solutions was allowed to occur for several minutes before recording commenced.

For capacitance measurements, cells were subjected to a repetitive protocol consisting of 10 s of sinusoidally oscillating holding potential (peak-to-peak amplitude = 32 mV; frequency = 1 kHz) around −60 mV, followed by a 500-ms ramp from −100 mV to +50 mV to assess membrane conductance. Membrane capacitance was calculated once per sine wave cycle with the software lock-in extension of the Pulse software (5) and then averaged over 100 sine wave cycles, yielding an effective capacitance sampling rate of ~10 Hz.

Carbamylcholine (carbachol, CCh; 10 \(\mu M\)), phenylephrine (PE; 10 \(\mu M\)), and ionomycin (28 \(\mu M\)) were dissolved in the external solution described above and applied by local superfusion through an application pipette with a tip diameter of 10–20 \(\mu M\) placed near the recorded cell. In the case of ionomycin, the position of the application pipette was adjusted empirically to slow the resulting rise in ([Ca\(^{2+}\)]\(_i\)), sufficiently to allow resolution of the calcium dependence of exocytosis.

Fluorescence measurements of ([Ca\(^{2+}\)]\(_i\)). Measurement of ([Ca\(^{2+}\)]\(_i\)), from fura 2 fluorescence was performed as detailed previously (11). Spatially averaged fluorescence measurements were made with a photomultiplier tube with a pinhole oriented to view the entire cell. Alternating excitation light at 360 and 390 nm was provided by a rotating filter wheel in the epifluorescence path of the inverted microscope. ([Ca\(^{2+}\)]\(_i\)) was calculated from the ratio of emitted light at the two excitation wavelengths with calibration constants obtained by dialyzing cells with solutions containing known, highly buffered concentrations of free calcium (11). For studies of the calcium dependence of the rate of exocytosis, only calcium levels up to 1,000 nM were analyzed.
cation of the cholinergic agonist CCh or the α1-adrenergic agonist PE transiently increased both \([\text{Ca}^{2+}]\), and membrane capacitance. The peak change in capacitance was not significantly different for the two agonists, averaging \(2.6 \pm 0.6 \text{ pF (mean \pm SE; } n = 27)\) in response to CCh and \(2.0 \pm 0.8 \text{ pF (} n = 14)\) after application of PE (see Fig. 1C). Cells were variable in size, with total cell capacitance ranging from 13 to 52 pF before agonist stimulation (average = 21.6 pF; \(n = 41\)). Because the number of secretory granules available for exocytosis, and hence the size of the capacitance response, would likely be greater in larger cells, we expressed the agonist-activated increase in capacitance as a percentage of the prestimulus baseline capacitance for each cell. On average, CCh increased the surface area of lacrimal gland acinar cells by 12.0 \(\pm 3.3\%\), which was not significantly different from the increase of 9.2 \(\pm 2.7\%\) evoked by PE.

Although the capacitance changes evoked by PE and CCh were similar in size, the rate of exocytosis differed for the two agonists. The rate of exocytosis evoked by agonist stimulation was estimated from the slope of the initial rise in capacitance, obtained by fitting a straight line to the first four data points at the foot of the response. The slope averaged \(3 \pm 1.5 \text{ pF/s (} n = 8\) for CCh-stimulated cells and \(0.8 \pm 0.3 \text{ pF/s (} n = 12\) for PE-stimulated cells. To account for variability in cell size and magnitude of secretory response, the initial slope was divided by the size of the capacitance response to obtain the rate constant for the initial rise of the capacitance response for each cell. The exocytosis rate averaged \(2 \pm 0.6 \text{ s}^{-1} \text{ (mean \pm SE; } n = 8)\) for CCh and \(0.5 \pm 0.1 \text{ s}^{-1} \text{ (} n = 12\) for PE (see Fig. 1C), which was a statistically significant difference \((P < 0.04; 2\text{-tailed } t\text{-test with unequal variances}). The calcium concentration during the initial rise of the capacitance response was similar for the two agonists, averaging \(241 \pm 55 \text{ nM for CCh and 279} \pm 40 \text{ nM for PE. Thus the difference in exocytosis rate cannot be explained by a difference in the spatially averaged calcium concentration.}

The capacitance increase stimulated by CCh or PE was followed by a slower return of capacitance to baseline, representing retrieval of the membrane added during the preceding burst of exocytosis. In addition, the capacitance baseline of single acinar cells typically fluctuated slowly even in the absence of agonist stimulation (see Fig. 1, A and B, insets). This fluctuation likely reflects ongoing basal membrane turnover (17). The ongoing membrane turnover and the stimulus-associated endocytosis may overlap with the exocytotic burst activated by CCh or PE so that estimates of exocytosis obtained from the capacitance rise may underestimate the true amount of added membrane. However, the rise time of the agonist-stimulated capacitance increase \((2.7 \pm 1.3 \text{ s, measured from the onset to the peak of the capacitance response})\) was rapid compared with the time required for poststimulus membrane retrieval \((15.6 \pm 7.8 \text{ s, measured from the beginning of the decline in capacitance to the poststimulus baseline})\), and baseline fluctuations were also slow compared with agonist-activated capacitance changes (see Fig. 1, A and B). Thus the underestimate of exocytosis caused by overlapping endocytosis may not be severe.

**Internal calcium concentration controls exocytosis in lacrimal gland acinar cells.** Changes in membrane capacitance activated by CCh and PE correlated well with the time course of changes in \([\text{Ca}^{2+}]\). For instance, in Fig. 1A, a second wave of calcium followed the first peak and a secondary rise in capacitance coincided with the second calcium wave. Also, both adrenergic and cholinergic agonists produced capacitance responses, although the signaling pathways from the receptor to calcium release are thought to differ for the two agonists (14). This suggests that elevation of internal calcium, the common feature of the signaling pathways, is the relevant feature for stimulation of exocytosis. Therefore, we investigated whether the change in \([\text{Ca}^{2+}]\) is necessary and sufficient for capacitance responses in lacrimal gland acinar cells.

If elevation of \([\text{Ca}^{2+}]\), drives exocytosis, then preventing the rise in \([\text{Ca}^{2+}]\), should block the capacitance response to CCh. To test this possibility, we dialyzed acinar cells with internal solution containing added EGTA to buffer changes in \([\text{Ca}^{2+}]\). Figure 2A shows that 1 mM EGTA was insufficient to prevent the increase in \([\text{Ca}^{2+}]\), and capacitance in response to CCh. However, as illustrated in Fig. 2B, increasing the con-

![Fig. 2. Buffering internal calcium with EGTA prevents the capacitance response elicited by CCh. A: the CCh response of a control cell dialyzed with 1 mM EGTA. B: the absence of calcium and capacitance responses in a cell dialyzed with internal solution containing 20 mM EGTA. The arrow indicates the timing of the CCh application for both experiments.](http://ajpcell.physiology.org/)
centration of EGTA to 20 mM prevented the calcium response and abolished the change in capacitance. Similar elimination of the capacitance response was observed in 4 cells with 20 mM EGTA and 12 cells with 10 mM EGTA. Thus elevation of \([Ca^{2+}]_i\) was necessary for exocytosis triggered by cholinergic stimulation.

We next determined whether elevation of \([Ca^{2+}]_i\) is sufficient to elicit exocytosis in the absence of agonist stimulation. To increase \([Ca^{2+}]_i\), the calcium ionophore ionomycin was applied by local superfusion of the recorded acinar cell. Figure 3 shows that a prominent increase in capacitance accompanies the elevation of \([Ca^{2+}]_i\). Capacitance responses evoked by ionomycin averaged 5.5 ± 0.4 pF (n = 17) compared with 2.6 ± 0.6 pF for CCh and 2.0 ± 0.8 pF for PE (see Fig. 1C). Thus capacitance responses similar to those stimulated by muscarinic and adrenergic agonists were observed when \([Ca^{2+}]_i\) was increased directly by application of a calcium ionophore.

We conclude that elevation of \([Ca^{2+}]_i\) is both necessary and sufficient for exocytosis in acinar cells. This result suggests that neural inputs trigger exocytosis in the lacrimal gland primarily by elevating \([Ca^{2+}]_i\) and that other aspects of the second messenger response to agonists are not required for the exocytotic response.

**Calcium dependence of rate of exocytosis.** The rise of both \([Ca^{2+}]_i\) and capacitance induced by ionomycin was relatively slow under the conditions of our experiments, which allowed the relation between the calcium level and the rate of rise of capacitance to be resolved as \([Ca^{2+}]_i\) increased. An example is illustrated in Fig. 4A, which shows that the capacitance response clearly lagged the rise of \([Ca^{2+}]_i\). The rate of increase of capacitance accelerated as calcium increased further, achieving its maximal rate as \([Ca^{2+}]_i\) approached saturation for the fura 2 indicator. The capacitance rise then decelerated as it reached a plateau level, which may reflect in part depletion of the pool of available secretory granules. To estimate the relation between

![Fig. 4. Calcium dependence of the rate of exocytosis in lacrimal gland acinar cells. A: an example of the calcium response (●) and capacitance change (solid line) elicited by application of the calcium ionophore ionomycin (28 \(\mu\)M). B: average exocytosis rate as a function of \([Ca^{2+}]_i\) during the rising phase of the capacitance response elicited by ionomycin. Each data point represents the mean of 6–12 observations averaged over the following \([Ca^{2+}]_i\) ranges: 0–100, 100–200, 200–300, 300–400, 400–500, and 500–950 nM. X and Y error bars represent ±1 SE. A single aberrant observation, resulting from a stepwise jump in capacitance that occurred in 1 instance within a single digitization interval, was not included in the averages. The solid line was drawn according to the equation rate = \(([Ca^{2+}]_i/725)^{1.2}\), which was fitted to the data by a least-squares criterion.](http://ajpcell.physiology.org/)

\([Ca^{2+}]_i\) and the rate of exocytosis at calcium concentrations <1,000 nM, we fitted a straight line to the change in capacitance within 1- to 2-s epochs during the accelerating portion of the rising phase of the capacitance response. To account for the progressive depletion of available secretory granules and to allow comparison across cells having different capacitance response amplitudes, the absolute rate in each epoch was divided by the amount of total capacitance response remaining (i.e., the difference between the peak capacitance and the average capacitance during the epoch). For an exponential process, this normalization procedure would yield the exponential rate constant. Within each epoch, average \([Ca^{2+}]_i\) was also calculated.

Figure 4B shows the relation between \([Ca^{2+}]_i\) and exocytosis rate obtained from ionomycin responses in 12 lacrimal gland acinar cells. The solid line represents the best-fitting power function, in which exocytosis rate is proportional to \([Ca^{2+}]_i\) raised to the power of 3.2. A similar power law has been reported for other secretory systems, such as neurotransmitter release from synaptic terminals (exponent ≈ 4; Ref. 1) and catecholamine release from adrenal chromaffin cells.

![Fig. 3. Elevating \([Ca^{2+}]_i\), with a calcium ionophore stimulates exocytosis. The increase in \([Ca^{2+}]_i\), elicited by superfusion with the calcium ionophore ionomycin (B) and the simultaneously recorded cell capacitance (A) are shown.](http://ajpcell.physiology.org/)
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(exponent = 3; Ref. 13). This relationship is consistent with models in which multiple calcium ions must bind to a sensor mechanism to trigger exocytosis of a secretory granule.

**DISCUSSION**

Activation of muscarinic and adrenergic receptors induces a transient increase in intracellular free calcium and stimulates secretion of protein in lacrimal gland acinar cells (7, 8, 16, 22). In most studies, these observations have been made with large groups of either isolated cells or gland fragments because the amount of protein secreted by a single cell is not easily measured. In our work, however, we were able to correlate intracellular free calcium with the secretory process in a single acinar cell by measuring the increase in cell membrane capacitance during exocytosis. Our results demonstrated that the increase in \([\text{Ca}^{2+}]_i\) that accompanies adrenergic or cholinergic activation is necessary to trigger exocytosis. Also, directly increasing \([\text{Ca}^{2+}]_i\) with a calcium ionophore effectively stimulated exocytosis in the absence of agonist stimulation. We conclude that the secretory process in lacrimal gland acinar cells is directly controlled by intracellular calcium, as it is in other secretory cells (10, 13, 25).

By measuring calcium concentration and exocytosis simultaneously in the same cell, we also were able to establish that the rate of secretion increased with approximately the third power of \([\text{Ca}^{2+}]_i\) in lacrimal gland acinar cells. This steep dependence on \([\text{Ca}^{2+}]_i\) suggests that at least three calcium ions are required to trigger calcium-dependent exocytosis. A similar steep dependence of exocytosis rate on calcium concentration has been reported for secretion in other cells, including chromaffin cells (13), pituitary gonadotrophs (25), and synaptic terminals (1, 10, 11), among others. Given the capacitance responses elicited when calcium was elevated with ionomycin (see Fig. 4B), exocytosis in lacrimal gland acinar cells is sensitive to low levels of \([\text{Ca}^{2+}]_i\), in the range <300 nM. However, levels exceeding 1 \(\mu\)M are required to trigger significant exocytosis in at least some other cell types (11, 25). This suggests that the calcium-sensing molecules in lacrimal gland acinar cells have relatively high affinity for calcium ions.

Secretory vesicles in acinar cells of the lacrimal gland are comparatively large, with diameters of 1 \(\mu\)m or more (23). The initial rate of rise of capacitance after CCh stimulation averaged 3 pF/s, which corresponds to the fusion of \(\sim 95\) 1-\(\mu\)m vesicles/s. In PE-stimulated cells, the initial rate of 0.8 pF/s would require fusion of \(\sim 25\) vesicles/s. These fusion rates are substantially higher than the rate of fusion of zymogen granules in pancreatic acinar cells, in which granules are estimated to fuse at a rate of 0.5 s\(^{-1}\) after cholinergic stimulation (6). The vesicle fusion rate in lacrimal gland acinar cells is more similar to that reported in chromaffin cells stimulated by bradykinin (2), which increases capacitance at 10–50 pF/s or \(\sim 4–20\) vesicles/s. However, chromaffin cells are capable of very high fusion rates (up to 40,000 vesicles/s) when secretion is driven by calcium influx through voltage-gated calcium channels or by photolysis of caged calcium, instead of agonist stimulation (12).

Peak capacitance responses like those we observed after agonist stimulation (average of 2 pF for PE and 2.6 pF for CCh) would require the full fusion of 60–80 1-\(\mu\)m vesicles. This relatively large capacitance change corresponds to \(\sim 10\%\) of the resting cell surface area. If a similar increase in surface area occurs in the cells in situ, it raises the question of where the membrane is added. The apical membrane of acinar cells, which faces the lumen of the duct in the intact gland, is a small proportion of the total surface membrane, and so an increase of 10% of the total membrane area added selectively at the apical surface would significantly distort the cell morphology. Because the acinar cells are coupled by junctional complexes, it is hard to see how such distortion could occur. One possibility is that the membrane fusion actually occurs by means of vesicles fusing with one another, creating an extended apical membrane that effectively intrudes within the cell rather than extending into the duct. Such a mechanism has been demonstrated to occur for the fusion of zymogen granules in pancreatic acinar cells (21). If it occurs in lacrimal gland acinar cells, vesicle-vesicle fusion could account for the relatively rapid fusion and retrieval of membrane we observed during and after agonist stimulation.

The stimulus-induced change in capacitance was superimposed on a baseline fluctuation in capacitance that varied in magnitude across cells (see Fig. 1, A and B). The temporal fluctuations in capacitance could be caused by random fusion and retrieval of secretory vesicles with the apical membrane, which could produce a basal rate of secretion independent of stimulation. Alternatively, the capacitance fluctuations could reflect the exocytic and endocytic activity of the basolateral membrane that has been described previously (17).

When \([\text{Ca}^{2+}]_i\) was elevated by cholinergic or adrenergic stimulation, the exocytosis rate at a calcium concentration of \(\sim 250\) nM was \(2\) s\(^{-1}\) after application of CCh and 0.5 s\(^{-1}\) in response to PE. By contrast, when \([\text{Ca}^{2+}]_i\) was elevated with ionomycin, the exocytosis rate achieved at 250 nM was only \(\sim 0.03\) s\(^{-1}\), which is substantially slower than the rate evoked by either agonist at comparable \([\text{Ca}^{2+}]_i\). From the power function shown in Fig. 4B, the exocytosis rate in response to CCh would require \([\text{Ca}^{2+}]_i\) of 900 nM, whereas 580 nM \([\text{Ca}^{2+}]_i\) would be required to produce the rate observed in response to PE. This disparity between the exocytosis rates observed with ionomycin on the one hand and CCh or PE on the other may indicate that the effective \([\text{Ca}^{2+}]_i\), that actually drives exocytosis during agonist stimulation is higher than the spatially averaged \([\text{Ca}^{2+}]_i\), calculated from our photometer measurements of fura 2 fluorescence. This might arise, for example, if CCh and PE release calcium in localized subcellular domains, resulting in pockets of higher \([\text{Ca}^{2+}]_i\), near the...
calcium sensors that trigger exocytosis, as has been suggested for calcium release in pituitary gonadotrophs (25).

In this view, the slower rate in response to PE may indicate that the calcium released by PE is less spatially localized than that released by CCh. By contrast, ionomycin shuttles calcium uniformly across the plasma membrane, producing a more spatially homogenous elevation of $[Ca^{2+}]_i$ that may be more accurately represented by our photometric calcium measurements. Alternatively, aspects of the second messenger cascades activated by cholinergic and adrenergic receptors may accelerate calcium-triggered exocytosis, resulting in slower exocytosis when calcium is elevated without activation of the receptors. Such second messenger actions could differ between PE and CCh, which may potentially explain why the two agonists induce different rates of exocytosis at comparable levels of intracellular calcium. It is also possible that a slow increase in $[Ca^{2+}]_i$, such as that produced by ionomycin, is less effective in stimulating exocytosis because of adaptation of the calcium-sensing machinery (15). At present, we cannot distinguish among these possible explanations for the different rates of exocytosis evoked by CCh, PE, and ionomycin.

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