Rapid Increases in Cytosolic Free Calcium in Response to Muscarinic Stimulation of Rat Parotid Acinar Cells*

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Carbachol-evoked rises in \([\text{Ca}^{2+}]_{\text{i}}\) were measured in fura-2-loaded, rat parotid acinar cells. In suspensions of dissociated cells examined by dual wavelength excitation fluorimetry, a maximally effective concentration of carbachol produced a measured peak \([\text{Ca}^{2+}]_{\text{i}}\), of 750 ± 60 nM followed by a maintained elevation in the presence of 1 mM external \(\text{Ca}^{2+}\), and a peak of 630 ± 95 nM followed by a return to resting values in the absence of external \(\text{Ca}^{2+}\). Stopped-flow, single wavelength fluorimetry was used to resolve the rising phase of the response. There was a dose-dependent lag of 70–220 ms before \([\text{Ca}^{2+}]_{\text{i}}\), started to increase, and \([\text{Ca}^{2+}]_{\text{i}}\), was maximal by 800–900 ms. These times were similar in the presence or absence of external \(\text{Ca}^{2+}\), although the initial rate of rise was faster in the presence of external \(\text{Ca}^{2+}\). These kinetics are consistent with a bimolecular event, possibly phosphatidylinositol bisphosphate hydrolysis, mediating both internal release and \(\text{Ca}^{2+}\) entry, with a component of the initial rise being due to \(\text{Ca}^{2+}\) entry.

The parotid gland has been extensively used as a model system for the study of \(\text{Ca}^{2+}\) regulation and its relation to inositol lipid hydrolysis (1, 2). Most studies to date have relied on measurement of \(\text{Ca}^{2+}\) fluxes (2) or of unidirectional \[^{86}\text{Rb}^+\] efflux (3) (\(\text{Ca}^{2+}\)-activated \(K^+\) channels) which provides only an indirect bioassay of \([\text{Ca}^{2+}]_{\text{i}}\) and a time resolution of minutes (1, 3). The highly fluorescent \(\text{Ca}^{2+}\) indicator dye, fura-2 (4), allows a direct, more rapid resolution of changes in \([\text{Ca}^{2+}]_{\text{i}}\), such that mixing time is the limiting factor in a conventional spectrofluorimeter; in response to muscarinic stimulation by carbachol, \([\text{Ca}^{2+}]_{\text{i}}\) is maximal within 1–2 s (see "Results," Fig. 1). Stopped-flow spectrofluorimetry allows resolution of changes in fluorescence and therefore \([\text{Ca}^{2+}]_{\text{i}}\), over the millisecond range (5). The main purpose of the studies reported here was to compare the kinetics of carbachol-stimulated increases in \([\text{Ca}^{2+}]_{\text{i}}\), in parotid acinar cells in the absence or presence of external \(\text{Ca}^{2+}\), to see how fast \(\text{Ca}^{2+}\) can be mobilized from internal stores, and to assess what part may be played by \(\text{Ca}^{2+}\) entry in the initial response. In the parotid gland, activation of muscarinic receptors by carbachol causes hydrolysis of phosphatidylinositol bisphosphate with the production of diacylglycerol and inositol phosphates, including (1,4,5)IP\(_3\) and inositol tetrakisphosphate (6, 7). We can thus place kinetic constraints on models based on receptor-mediated hydrolysis of phosphatidylinositol bisphosphate and a trigger role for inositol phosphates (8–14).

**MATERIALS AND METHODS**

Fura-2 acetoxyethyl ester was obtained from Molecular Probes Inc., Eugene, OR, collagenase from Boehringer Mannheim and all other reagents from Sigma or British Drug House.

Acinar cells were prepared from parotid glands of male Wistar rats (150 g) by modifications of the methods of Putney et al. (11) and Merritt et al. (12). The basic medium contained NaCl (107 mM), KCl (6 mM), Na\_2HPO\_4 (1.2 mM), Hepes (20 mM), pH 7.4, MgSO\_4 (1.2 mM, unless otherwise stated), CaCl\_2 (1 mM, unless otherwise stated), glucose (11.5 mM), and essential amino acids. For cell preparation, the medium contained bovine serum albumin (0.65%) and 

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for experiment with dispersed cells. The minced glands were washed in medium, then incubated 15 min at 37 °C with medium containing trypsin (0.5 mg/ml). Medium containing EDTA (2 mM) and no Ca\(^{2+}\) or Mg\(^{2+}\) was used for the next stage, 5 min with soybean trypsin inhibitor (0.5 mg/ml), then 5 min in fresh medium without trypsin inhibitor. Tissue was then incubated a further 15–20 min in normal medium containing collagenase (0.35 mg/ml), after which cells were dispersed by vigorous shaking, filtered through gauze, and centrifuged for 5 min at 50 g. These cells were resuspended in normal medium, layered over medium containing 4% bovine serum albumin, and again centrifuged for 5 min at 50 g. Cell viability, assessed by trypan blue exclusion, was >98%.
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Cells were resuspended in medium containing albumin (0.1%) and fura-2 A/M (2 μM) and incubated for 45 min at 37 °C. These incubation conditions resulted in an intracellular concentration of about 35 μM fura-2. Following centrifugation (5 min at 50 g), cells were resuspended at twice the final density in medium containing albumin (0.1%). Fura-2 fluorescence of these cells was measured in a Hi-Tech (Salisbury, Wilts, U.K.) stopped-flow spectrofluorimeter, with excitation at 340 nm and emission at 505 nm (cut-off filter). In this system, cells were rapidly mixed with agonist (also made at twice the final concentration in the same medium), and recording of fluorescence was triggered with a delay of <5 ms. Fluorescence was continuously monitored for 1 s, with a filter time constant of 10 ms, and the data were stored on an Apple microcomputer. Traces from 4–6 runs were averaged, and an averaged control (i.e. no agonist) run from the same batch of cells was subtracted to eliminate any artifacts due to cell damage from shear forces. The change in fluorescence in these control runs in the presence of 1 mM \(\text{Ca}^{2+}\) was about 20% of the total change in fluorescence observed in response to carbachol (1 mM). There was no change in fluorescence in control runs in the presence of EGTA, as any leaked dye would give only a minimal signal. For experiments in the absence of external \(\text{Ca}^{2+}\), EGTA (3 mM) was added to aliquots of cells and agonist just before use. In each experiment, times were measured from the traces to the nearest 10 ms for lag times and half-maximal times and 50 ms for peak times.

For actual measurement of \([\text{Ca}^{2+}]_{\text{i}}\), the fluorescence of fura-2-loaded cells was measured in a Spex dual wavelength fluorimeter (Glen Creston Instruments Ltd., Stanmore, Midddx, U.K.). \([\text{Ca}^{2+}]_{\text{i}}\) was calculated from the ratio of fluorescence (500-nm emission) at two excitation wavelengths, 340 and 380 nm, as described by Grynkiewicz et al. (4).

**RESULTS**

Fig. 1 shows the effect of a maximal concentration of carbachol (1 mM) on \([\text{Ca}^{2+}]_{\text{i}}\), in parotid acinar cells in the
Fig. 1. Effect of carbachol on [Ca\textsuperscript{2+}]i in fura-2-loaded parotid acinar cells. [Ca\textsuperscript{2+}]i was calculated from the fluorescence at two excitation wavelengths (340 and 380 nm). Carbachol (CCh, 1 mM) was added, at the times indicated, to cells in the presence of Ca\textsuperscript{2+} (1 nM) or with EGTA (3 mM) added. These traces are typical of four independent cell preparations.

Fig. 2. Carbachol stimulates rapid increases in fluorescence of fura-2-loaded parotid acinar cells. Each trace is the average of 4–6 runs from the same cell preparation with the appropriate control subtracted. The solid lines are from experiments in the presence of Ca\textsuperscript{2+} (1 mM), and the broken lines are with EGTA (3 mM) added. Fluorescence is shown in arbitrary units and not calibrated to [Ca\textsuperscript{2+}]i, although an increase in fluorescence corresponds to an increase in [Ca\textsuperscript{2+}]i. These traces are typical of four independent cell preparations.

Presence or absence of external Ca\textsuperscript{2+}. In four independent experiments, carbachol elevated [Ca\textsuperscript{2+}]i from a resting level of 195 ± 9 nM to a peak of 780 ± 60 nM in the presence of external Ca\textsuperscript{2+} (1 mM) and in the absence of external Ca\textsuperscript{2+} (1 mM Ca\textsuperscript{2+} plus 3 mM EGTA) from 175 ± 8 nM to a peak value of 630 ± 95 nM (means ± S.E.). In the absence of external Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]i returned to the resting level within 2 min after addition of carbachol, while in the presence of external Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]i remained elevated at a maintained level of 470 ± 20 nM, after the initial peak. It is clear that [Ca\textsuperscript{2+}]i has peaked within 1–2 s after addition of carbachol, either in the presence or absence of external Ca\textsuperscript{2+}, and that resolution of the time to peak is limited by the mixing time when making additions to a cuvette.

A representative set of averaged stopped-flow fluorescence traces showing changes in fura-2 fluorescence of rat parotid acinar cells in response to various concentrations of carbachol is shown in Fig. 2. These traces show fluorescence in arbitrary units and are not calibrated in absolute [Ca\textsuperscript{2+}]i; an increase in fluorescence at 340 nm (excitation) represents an increase in [Ca\textsuperscript{2+}]i. (In parallel experiments, when the excitation wavelength was set to 380 nm, a corresponding decrease in fluorescence was observed.) There was a clear lag before the onset of an increase in fluorescence, and this lag was dose-dependent. The fluorescence was maximal within 1 s. This increase in fluorescence was reversible; when fluorescence was monitored for the first 20 s, it started to decrease (not shown), which correlates with Fig. 1 where [Ca\textsuperscript{2+}]i decreased after the initial peak. The lag times and times to peak are summarized in Table I, which shows results combined from four separate experiments. From Fig. 2 and Table I, it is evident that both the lag times and the times to peak were similar in both the presence and absence of external Ca\textsuperscript{2+}. However, as shown in Fig. 2, the shapes of the curves differed, depending on whether or not external Ca\textsuperscript{2+} was present: in the presence of external Ca\textsuperscript{2+} (1 mM), the initial rate of rise in fluorescence was more rapid. This difference is quantified in Table I, which shows the times taken for a half-maximal rise in fluorescence, as measured from the beginning of the rise to 50% of the maximal response.

**DISCUSSION**

Carbachol stimulated a rapid increase in [Ca\textsuperscript{2+}]i in parotid acinar cells, as measured by the fluorescence of trapped fura-2. In the absence of external Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]i rapidly peaked and returned to resting levels within 2 min. This is presumably due to mobilization of Ca\textsuperscript{2+} from an intracellular store, which then becomes depleted as Ca\textsuperscript{2+} is extruded from the cells. In the presence of external Ca\textsuperscript{2+}, the initial peak [Ca\textsuperscript{2+}]i was slightly higher and was then followed by a maintained elevated level; this maintained elevation in [Ca\textsuperscript{2+}]i is presumably due to Ca\textsuperscript{2+} entry. These results provide direct evidence for carbachol-stimulated changes in [Ca\textsuperscript{2+}]i, which are consistent with the deductions made by Putney (3) from 86Rb+ fluxes.

Use of a stopped-flow spectrofluorimeter allowed us to make rapid measurements of the initial change in [Ca\textsuperscript{2+}]i, within the first second of stimulation by carbachol. In the absence of external Ca\textsuperscript{2+}, there was a measurable dose-dependent lag time of at least 80 ms before [Ca\textsuperscript{2+}]i began to increase; this lag may reflect the time required for accumulation of sufficient (1,4,5)IP3 to cause mobilization of Ca\textsuperscript{2+} from the endoplasmic reticulum.
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reticulum, although other possibilities cannot be excluded. Even at concentrations of carbachol which only cause a small (less than half-maximal) increase in \([Ca^{2+}]\), \(3\), and a small increase in IP\(_3\), \(13\), the lag time before \([Ca^{2+}]\) rises was only 200 ms. We know of no measurements of \(1,4,5\)IP\(_3\) formation within the first second of stimulation of any preparation; the shortest times reported for parotid gland are 5 s, when \((1,4,5)IP_3\) is already substantially increased \((6, 7)\). Clearly such measurements are urgently needed since our data, and the generally accepted hypothesis, require that \((1,4,5)IP_3\) should be elevated within 100 ms of stimulation.

The lag time was not measurably different in the presence or absence of external Ca\(^{2+}\) and \([Ca^{2+}]\), peaked around the same time. This result contrasts with that reported for thrombin-stimulated platelets \((5)\), where Ca\(^{2+}\) influx seems to precede internal mobilization, judged from the shorter lag time seen in the presence of external Ca\(^{2+}\). However, in the parotid, the initial rate of rise in \([Ca^{2+}]\), was distinctly more rapid in the presence of external Ca\(^{2+}\) and the peak \([Ca^{2+}]\), was slightly higher, pointing to a Ca\(^{2+}\) influx component at these early times, in addition to that at later times when a sustained Ca\(^{2+}\) entry was observed. The results in parotid cells could be explained if both internal release and entry of Ca\(^{2+}\) are controlled by the same biochemical event, probably phosphatidylinositol bisphosphate hydrolysis. It is not easy to fit our results with the “capacitative model” for Ca\(^{2+}\) entry proposed by Putney \((9)\). In this model, \((1,4,5)IP_3\) mediates internal release, and entry is consequent upon depletion of the dischargeable pool; thus, one would not predict that \([Ca^{2+}]\), would initially rise more rapidly when Ca\(^{2+}\) entry is possible. (It should be pointed out that the faster rise in the presence of external Ca\(^{2+}\) might reflect more effective signal transduction, rather than an additional Ca\(^{2+}\) entry. The fact that, in many preparations, including parotid cells, IP\(_3\) formation is not affected by removal of external Ca\(^{2+}\) \((13)\), may argue against this.)

In summary, we have demonstrated that, in parotid acinar cells, \([Ca^{2+}]\), can be elevated, by a combination of internal release and influx, within 100 ms of exposure to carbachol. The proposed transduction pathways must, therefore, be shown to be operative within this time.

REFERENCES

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\(^{1}\) J. E. Merritt and T. J. Rink, unpublished observations.