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# Physiological localization of an agonist-sensitive pool of $Ca^{2+}$ in parotid acinar cells

[stimulus-secretion coupling/fura-2/salivary epithelial cells/intracellular $Ca^{2+}$ stores/bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate]

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ABSTRACT **Muscarinic stimulation of fluid secretion by mammalian salivary acinar cells is associated with a rise in the level of intracellular free calcium ($[Ca^{2+}]_i$) and activation of a calcium-sensitive potassium ($K^+$) conductance in the basolateral membrane. To test in the intact cell whether the rise of $[Ca^{2+}]_i$ precedes activation of the $K^+$ conductance (as expected if $Ca^{2+}$ is the intracellular messenger mediating this response), $[Ca^{2+}]_i$ and membrane voltage were measured simultaneously in carbachol-stimulated rat parotid acinar cells by using fura-2 and an intracellular microelectrode. Unexpectedly, the cells hyperpolarize (indicating activation of the $K^+$ conductance) before fura-2 detectable $[Ca^{2+}]_i$ begins to rise. This occurs even in $Ca^{2+}$-depleted medium where intracellular stores are the only source of mobilized $Ca^{2+}$. Nevertheless, when the increase in $[Ca^{2+}]_i$ was eliminated by loading cells with the $Ca^{2+}$ chelator bis(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate ($Me_2$BAPTA) and stimulating in $Ca^{2+}$-depleted medium, membrane hyperpolarization was also eliminated, indicating that a rise of $[Ca^{2+}]$ is required for the agonist-induced voltage response. Stimulation of $Me_2$BAPTA-loaded cells in $Ca^{2+}$-containing medium dramatically accentuates the temporal dissociation between the activation of the $K^+$ conductance and the rise of $[Ca^{2+}]_i$. The data are consistent with the hypothesis that muscarinic stimulation results in a rapid localized increase in $[Ca^{2+}]_i$ at the acinar basolateral membrane followed by a somewhat delayed increase in total $[Ca^{2+}]_i$. The localized increase cannot be detected by fura-2 but is sufficient to open the $Ca^{2+}$-sensitive $K^+$ channels located in the basolateral membrane. We concluded that a receptor-mobilized intracellular store of $Ca^{2+}$ is localized at or near the basolateral membrane.**

Fluid secretion by mammalian exocrine cells, including salivary acinar cells, is regulated by alterations in plasma membrane ion permeabilities (1). Among the earliest events associated with agonist-induced stimulation of fluid secretion are a rapid plasma membrane hyperpolarization (1–4) and a dramatic loss of cellular $K^+$ (5). Patch-clamp studies have identified $Ca^{2+}$- and voltage-sensitive $K^+$ channels in salivary and pancreatic acinar basolateral membranes whose activation by secretagogues is believed to underlie these effects (6). Another early event associated with agonist stimulation is a rapid rise in the level of intracellular free calcium ($[Ca^{2+}]_i$) (7–11). As in a wide variety of other cell types, $[Ca^{2+}]_i$ is thought to rise as a result of inositol trisphosphate mobilization of intracellular stores (9, 12, 13), as well as enhanced $Ca^{2+}$ influx across the plasma membrane (7, 14–17). A large body of evidence indicates that the rise of $[Ca^{2+}]_i$ is directly responsible for activating the $K^+$ channels in the acinar basolateral membranes. Specifically, it has been shown that (*i*) the $Ca^{2+}$ sensitivity of the $K^+$ channels is within the appropriate physiological (agonist-induced) range of $[Ca^{2+}]_i$ (6, 18–21), (*ii*) the cellular $K^+$ loss is abolished in $Ca^{2+}$-depleted cells stimulated in $Ca^{2+}$-free medium (22, 23), (*iii*) the application of $Ca^{2+}$ ionophores to acinar cells activates $K^+$ channels and causes a loss of cellular $K^+$ similar to that caused by agonists (23–26), (*iv*) the intracellular perfusion of a $Ca^{2+}$ chelator blocks the agonist-stimulated increase in $K^+$ conductance (18, 26, 27), and (*v*) the intracellular perfusion of inositol trisphosphate activates the $K^+$ conductance (28).

One aspect that has not yet been explored, however, is the temporal relationship between the agonist-induced rise of $[Ca^{2+}]_i$ and the activation of the basolateral membrane $K^+$ conductance. If $Ca^{2+}$ is indeed the relevant intracellular messenger, one would expect the rise in $Ca^{2+}$ to precede the response that it signals. Recently, a new generation of $Ca^{2+}$ indicator dyes has made it possible to measure agonist-induced changes in $[Ca^{2+}]_i$ with high temporal resolution in single cells (29). In the present study, we examine the relationship between the agonist-induced rise of $[Ca^{2+}]_i$ (measured with the $Ca^{2+}$-sensitive fluorescent dye fura-2) and membrane $K^+$ conductance (measured simultaneously with an intracellular microelectrode) in rat parotid acinar cells. The data indicate that a receptor-mobilized store of $Ca^{2+}$ is localized at or near the basolateral membrane.

## MATERIALS AND METHODS

**Parotid Acinar Cell Preparation.** Parotid acini from Wistar strain rats (euthanized by exsanguination while under general anesthesia) were isolated by collagenase/hyaluronidase digestion (30). A light fraction consisting of the pooled supernates from three 30-sec centrifugations (400 × *g*) of this preparation was used in the present studies. This fraction consisted of single cells, doublets, triplets, single acini, and small clumps of acini, as well as fragments of ducts. This material was suspended in albumin-free medium and plated onto glass coverslips.

**Fura-2 and BAPTA Loading.** Cells and aggregates adhering within 90 sec following plating were loaded with fura-2 (Molecular Probes) by incubation in 1.5 μM fura-2 acetoxymethyl ester at 37°C for 30 min in 5 ml of normal $Ca^{2+}$ medium (see below). The volume associated with cells that adhered to a single coverslip was negligible compared to this volume of bathing medium. Based on previous work (7, 8), this incubation resulted in an intracellular fura-2 concentration of 10–20 μM. Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate

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Abbreviations: BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate; $Me_2$BAPTA, 5,5′-dimethyl-BAPTA; DIC, differential interference contrast.

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(BAPTA) loading was accomplished by first loading the cells with fura-2 as described, then incubating the cells for an additional 90 min in 15 $\mu$M of the permeant acetoxymethyl ester of 5,5'-dimethyl-BAPTA ($Me_2$BAPTA; Molecular Probes) under identical conditions. The rationale for this procedure was based on two observations. First, cells loaded with fura-2 and examined 2 hr later were only slightly dimmer than cells examined immediately after loading (J.K.F., unpublished observations), indicating slow dye leakage. Second, the $[Ca^{2+}]_i$ responses of these cells were indistinguishable from freshly loaded cells, indicating that most of the dye remained in the cytoplasm as the $Ca^{2+}$-sensitive free acid. Fura-2 was not loaded simultaneously with BAPTA to avoid competition for cellular esterases by BAPTA, which was at a much higher concentration than the fura-2.

**Microscopy and Fluorescence and Electrophysiological Measurements.** The coverslip was mounted in a chamber and perfused continuously (bath volume = 50 $\mu$l; flow rate $\approx$ 4 ml/min) with a medium containing 110 mM NaCl, 5.4 mM KCl, 25 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.4 mM $KH_2PO_4$, 0.33 mM $NaH_2PO_4$, 20 mM Hepes, 1.2 mM $CaCl_2$, 11 mM glucose, 2 mM glutamine, and 0.01% bovine albumin (pH 7.4) at 37°C in 95% $O_2$/5% $CO_2$ on the stage of an inverted microscope (Zeiss IM35). EGTA (1 mM) replaced the $CaCl_2$ in zero-$Ca^{2+}$ medium. Membrane voltage was measured with respect to bath-ground with a glass microelectrode (100–200 M$\Omega$, filled with 0.5 M KCl/10 mM potassium citrate in HPLC-grade water) coupled to an amplifier (750, World Precision Instruments, New Haven, CT). Voltage was recorded on a chart recorder and displayed on the screen of an oscilloscope. The microelectrode was positioned and impalements were performed with a stepper motor-driven micromanipulator while viewing the cell in differential interference contrast (DIC) optics by using a ×63 oil-immersion 1.25 numerical aperture objective lens. The microscope is designed to allow simultaneous DIC imaging and low-light level fluorescence measurements without the light losses normally associated with DIC (31). A video camera viewed the image in DIC optics and relayed its signal to a monitor and to a video mixer. Fluorescence excitation was accomplished by placing interference filters (350 and 380 nm; ±10 nm bandwidth) between each of two xenon arc lamps coupled to a rotating chopper wheel mounted on a separate table to prevent vibration transmission to the microelectrode. A shutter between the chopper wheel and the microscope limited the duration of fluorescence excitation to minimize probe photobleaching and photodynamic damage to the cells. The chopper alternately exposed the cells to 350 nm and 380 nm excitation at 60 Hz. Fluorescence emission (500 ± 40 nm) was detected by a photomultiplier tube coupled to a current-to-voltage converter whose output was displayed on the oscilloscope screen. Stray light was minimized by conducting all experiments in red room light. In most experiments, an aperture was placed in an intermediate image plane to collect fluorescence emission only from the cell impaled with the electrode. The oscilloscope was line triggered; thus, the photomultiplier tube output appeared as two stationary peaks on the oscilloscope screen: the peaks are a measure of fluorescence emission at 350 and 380 nm excitation, respectively. A second video camera (newvicon; response time = 20 msec) viewed the oscilloscope screen and relayed its signal to the video mixer. The DIC image of the cell was inserted into the image of the oscilloscope screen allowing the simultaneously determined photomultiplier tube and microelectrode outputs and the image of the cell to be viewed side-by-side as one video output on a television monitor. This video signal was recorded at 15 Hz on a video disk recorder. This frequency represents the temporal resolution of these experiments since the response time of the electrode amplifier, photomultiplier tube, and oscilloscope are all much faster than this. Also, the response time of the newvicon tube is sufficiently fast (50 Hz) to avoid significant lag in the measured response. With regard to this latter point, any lag associated with the newvicon tube will affect the amplifier and photomultiplier tube outputs equally, since both are imaged by the newvicon. In many instances, the rapidity of the voltage and $[Ca^{2+}]_i$ changes observed in response to stimulation caused dual traces of each output to appear in the captured video frame, representing the temporal difference (16 msec) in acquisition of each video field. In these cases, the video field displaying the first detectable change was used for analyses. In most experiments, the transmitted light was shuttered off during fluorescence measurement to ensure against spectral contamination of the fura-2 signal. The data were analyzed by transfer of images from the video disk recorder to an image processing system (31). Fluorescence ratios (350/380 nm) were determined by division of background-subtracted peaks of emission at each excitation wavelength determined by using interactive software.

When fura-2 fluorescence was monitored during microelectrode penetrations in $Ca^{2+}$ medium, most impalements resulted in some increase in the 350 nm/380 nm ratio, indicating that $[Ca^{2+}]_i$ had risen. This was not observed in cells in zero-$Ca^{2+}$ medium, indicating that the rise in $[Ca^{2+}]_i$ was the result of $Ca^{2+}$ leaking into the cell around the edge of the microelectrode. Successful impalements were less likely in single cells and doublets compared to cells in single acini or acinar clumps, as has been observed by others (18). In several cells, it was possible to achieve an impalement in $Ca^{2+}$ medium with no or little evidence of a rise in $[Ca^{2+}]_i$. In these cells, the early responses of $[Ca^{2+}]_i$ and membrane voltage to stimulation by carbachol were indistinguishable from cells stimulated in zero-$Ca^{2+}$ medium.

**Fura-2 Calibration.** Fluorescence ratios were converted to $[Ca^{2+}]_i$ by calibration of a separate group of cells. After determination of the control (resting) ratio $R$, the cells were perfused with 1.5 $\mu$M ionomycin in a solution containing 140 mM KCl, 20 mM Hepes, 0.1 mM carbachol, and 5 mM EGTA (pH 7.3) at 37°C. When the limiting ratio for the unbound form of fura-2 was determined ($R_{min}$), the perfusate was switched to a similar one in which 2.4 mM $CaCl_2$ replaced the EGTA, and the limiting ratio of the bound form ($R_{max}$) was determined. $[Ca^{2+}]_i$ was calculated by using the equation $[Ca^{2+}]_i = \beta \cdot K_d[(R - R_{min})/(R_{max} - R)]$, where $K_d$ (the dissociation constant of the dye) was assumed to be 220 nM and $\beta$ is the ratio of fluorescence at 380 nm excitation for fura-2 (unbound)/fura-2 (bound) (29). For $n = 6$ cells, $\beta = 7.1$ and $R_{max}/R_{min} = 10$. Calculated resting $[Ca^{2+}]_i = 46 \pm 4$ nM. For the purposes of calibrating the cells used in experiments, resting $[Ca^{2+}]_i$ was assumed to equal this value, and this value with the derived $\beta$ and $R_{max}/R_{min}$ values was used to calculate $[Ca^{2+}]_i$ during stimulation. For $Me_2$BAPTA-loaded cells, the 350 nm/380 nm ratio was converted to $[Ca^{2+}]_i$ based on similar calibrations of a separate group of $Me_2$BAPTA-loaded cells. Average resting $[Ca^{2+}]_i$ in $Me_2$BAPTA-loaded cells was 41 nM. For both groups of cells, the length of time between loading and calibration was comparable to that between loading and experimentation.

## RESULTS

The muscarinic agonist carbachol typically caused a rapid ($t_{1/2} \approx 600$ msec) hyperpolarization (by 10–50 mV) of rat parotid acinar cells in both $Ca^{2+}$ and zero-$Ca^{2+}$ (0 mM $Ca^{2+}$/1 mM EGTA) medium (Fig. 1*A*). Membrane hyperpolarization by muscarinic agonists has been previously observed (2–4) and, as already mentioned, is thought to be mediated by activation of large-conductance $K^+$ channels (6). Simultaneous determinations of $[Ca^{2+}]_i$ and membrane potential in rat parotid acinar cells (Fig. 1*B*) demonstrated that the rapid

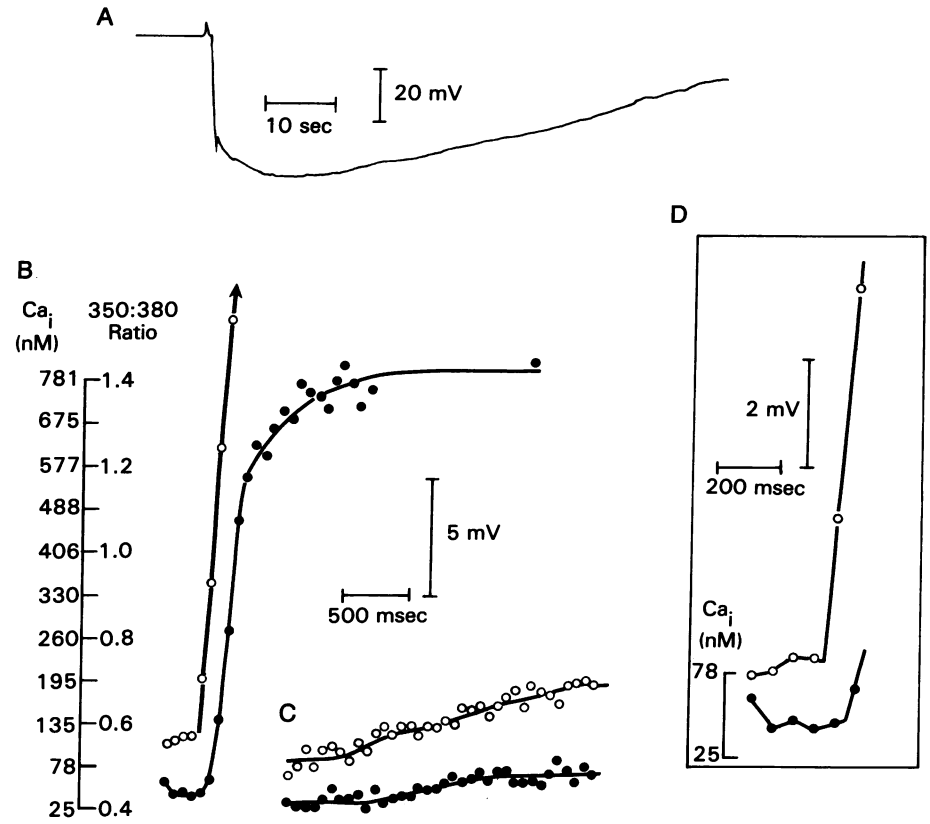


FIG. 1. Responses of $[Ca^{2+}]_i$ and membrane voltage in a rat parotid acinar during muscarinic stimulation. (*A*) Response of membrane voltage to rapid exposure to 1 mM carbachol in zero-$Ca^{2+}$ medium. Downward deflection indicates membrane hyperpolarization. Cells stimulated in $Ca^{2+}$ medium exhibited similar rapid hyperpolarizations but not the subsequent relaxation toward baseline voltage. Resting membrane potential = −28 mV; peak voltage = −78 mV. (*B*) Same data as in *A* at greater temporal resolution (15 Hz), along with simultaneously determined $[Ca^{2+}]_i$ measured with fura-2. Fura-2 350 nm/380 nm excitation ratios have been converted to $[Ca^{2+}]_i$ (in nM) according to calibration procedures described in *Materials and Methods*. Hyperpolarization is shown as an upward deflection to facilitate comparison with the fura-2 signal. (*C*) Same cell as in *A* and *B* showing responses of membrane voltage and $[Ca^{2+}]_i$ to a second application of 1 mM carbachol ≈2 min following removal of carbachol from the first exposure. (*D*) Same data as in *B* with expanded scales. ●, $[Ca^{2+}]_i$; ○, membrane voltage.

agonist-induced membrane hyperpolarization is paralleled by an equally rapid rise in $[Ca^{2+}]_i$ from ≈50 nM to ≈700 nM ($t_{1/2}$ < 500 msec). Removal and subsequent reapplication of the agonist a few minutes later elicited comparable responses in cells in $Ca^{2+}$ medium (data not shown), but cells stimulated a second time in zero-$Ca^{2+}$ medium exhibited a weak membrane hyperpolarization and only a small associated rise in $[Ca^{2+}]_i$ (Fig. 1*C*). Thus, the agonist-induced elevation of $[Ca^{2+}]_i$ and membrane hyperpolarization parallel each other both temporally and quantitatively. This suggests that the magnitude and kinetics of $[Ca^{2+}]_i$ determine the magnitude and kinetics of the membrane voltage responses, as expected if $[Ca^{2+}]_i$ is the intracellular activator of the $K^+$ channels. However, close examination of the temporal relationship between $[Ca^{2+}]_i$ and membrane voltage (Fig. 1*D*) in cells stimulated in either $Ca^{2+}$ or zero-$Ca^{2+}$ media revealed that the earliest detectable change in membrane potential typically preceded the earliest detectable increase in $[Ca^{2+}]_i$ by at least 67 msec (range, 0–200 msec; mean ± SEM = 80 ± 12 msec; $n$ = 23), the resolution of our apparatus (see *Materials and Methods*). This result is unexpected since a rise of $[Ca^{2+}]_i$ should precede the hyperpolarization if indeed $[Ca^{2+}]_i$ is the intracellular messenger activating the basolateral $K^+$ conductance.

One possible explanation for the above result is that $Ca^{2+}$ is not the relevant intracellular messenger. To confirm that a rise of $[Ca^{2+}]_i$ is necessary to elicit the agonist-induced hyperpolarization, cells were loaded with the $Ca^{2+}$ chelator $Me_2BAPTA$ ($K_d$ = 40 nM) (32) and stimulated in zero-$Ca^{2+}$ medium. Under these conditions, the only source of $Ca^{2+}$ that can be mobilized by carbachol will be intracellular stores. Provided the cells have been loaded with sufficient $Me_2BAPTA$, stimulation should result in a diminished $Ca^{2+}$ response. In 8 of 10 $Me_2BAPTA$-loaded cells tested, the agonist-induced increase of $[Ca^{2+}]_i$ and membrane hyperpolarization were both abolished (data not shown). In the 2 other cells, carbachol elicited slow hyperpolarizations from 2 to 5 mV (Fig. 2*A*), associated with elevation of $[Ca^{2+}]_i$ by only ≈30 nM over several seconds to a minute (data not shown).

Taken together with previous data (6, 18–28), these results indicate that a rise in $[Ca^{2+}]_i$ is in fact necessary to activate basolateral membrane $K^+$ conductance. The blunted response of $Me_2BAPTA$-loaded cells (Fig. 2*A*) is not due to nonspecific toxic effects of $Me_2BAPTA$ itself (33, 34) since, when $Me_2BAPTA$-loaded cells in zero-$Ca^{2+}$ medium were exposed for a brief period to $Ca^{2+}$ medium (3 min) following stimulation (Fig. 2*B*), a second application of carbachol in zero-$Ca^{2+}$ medium elicited a normal rapid hyperpolarization

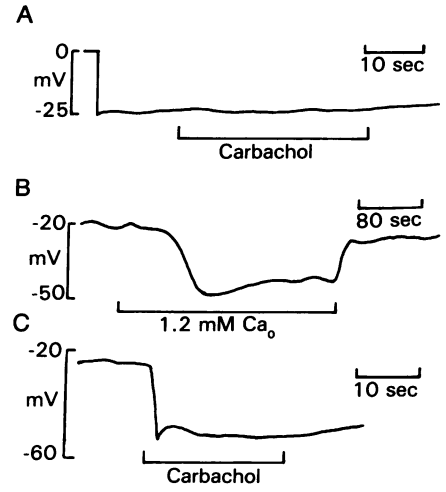


FIG. 2. Voltage responses of $Me_2BAPTA$-loaded cell stimulated in zero-$Ca^{2+}$ medium. A $Me_2BAPTA$-loaded cell was impaled (rapid deflection at beginning of trace) in zero-$Ca^{2+}$ medium. The cell was exposed to 1 mM carbachol (*A* and *C*) or 1.2 mM $Ca^{2+}$ (*B*) as indicated. The traces in *A–C* are a continuous record. As observed by others (4), exposure to $Ca^{2+}$ medium in the absence of carbachol (*B*) caused the membrane to hyperpolarize; the ionic basis for this effect is unknown. It was not associated with a fura-2-detectable rise in $[Ca^{2+}]_i$ (data not shown).

(Fig. 2*C*), demonstrating that $Me_2BAPTA$-loaded cells possess intact signal transduction processes.

An explanation that reconciles the seemingly contradictory observations that activation of the $K^+$ permeability requires a rise of $[Ca^{2+}]_i$ and yet precedes that rise is that, during the earliest times of stimulation, the plasma membrane senses a region of locally elevated $[Ca^{2+}]$, which precedes the increase in whole cell $[Ca^{2+}]_i$ measured by fura-2. To explore this possibility, we measured the responses of $Me_2BAPTA$-loaded cells stimulated in $Ca^{2+}$ medium. Under these conditions, in addition to the finite amount of $Ca^{2+}$ that can be mobilized from intracellular stores, the cytoplasm will have access to an essentially infinite extracellular pool of $Ca^{2+}$ as a result of the agonist-induced increase in plasma membrane $Ca^{2+}$ permeability (7, 8, 14–17). If this $Ca^{2+}$ influx is great enough, one would expect the eventual saturation of intracellular $Me_2BAPTA$ and a consequent rise in $[Ca^{2+}]_i$. Since $Ca^{2+}$ influx is at the basolateral membrane, the $Me_2BAPTA$ near the basolateral membrane might be expected to saturate before the $Me_2BAPTA$ in the bulk cytoplasm does. As a result, one would predict that $[Ca^{2+}]_i$ would rise first at the membrane, followed by a rise in the rest of the cytoplasm, which would be slowed by the presence of the chelator. Since the membrane hyperpolarization presumably depends on a rise of $[Ca^{2+}]_i$ adjacent to the inner surface of the basolateral membrane, under appropriate $Me_2BAPTA$ loading conditions, the cell should hyperpolarize significantly earlier than the fura-2-detected rise of $[Ca^{2+}]_i$. This is in fact exactly what is observed. Stimulation of $Me_2BAPTA$-loaded cells in $Ca^{2+}$ medium resulted in a large membrane hyperpolarization (Fig. 3) ($\Delta V = 26 \pm 4$ mV; $n = 9$ cells). Particularly striking, however, was the significant delay between the onset of the hyperpolarization and the earliest rise in $[Ca^{2+}]_i$. Generally, the earliest detectable increase in $[Ca^{2+}]_i$ occurred after the membrane completely hyperpolarized (Fig. 3*A*). In some cells (3 out of 9), $[Ca^{2+}]_i$ began to rise ≈300–1500 msec after the initial hyperpolarization, although the rate and magnitude of this increase were considerably diminished compared to unloaded cells (Fig. 3*A*). In other cells (4 out of 9), $[Ca^{2+}]_i$ remained unchanged during the first several seconds following the initial membrane hyperpolarization (Fig. 3*B*). In one of these cells for which a longer time course was followed, $[Ca^{2+}]_i$ eventually rose to higher levels (≈300 nM) by 75 sec. In two remaining cells, the responses of membrane potential and $[Ca^{2+}]_i$ were similar to those in unloaded cells.

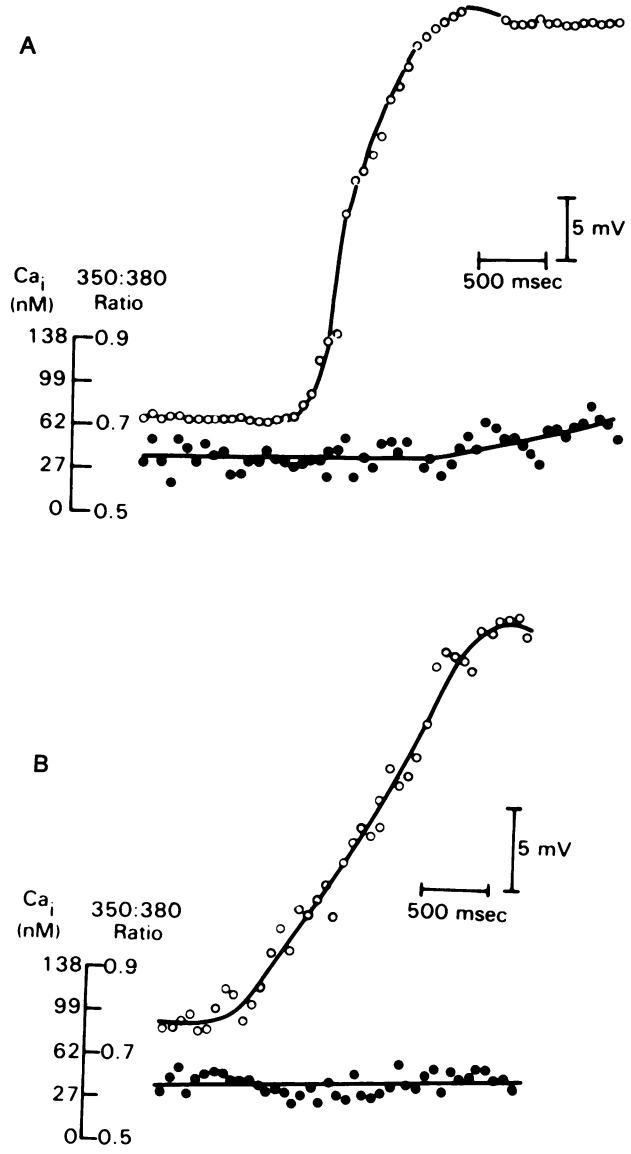


FIG. 3. Responses of $[Ca^{2+}]_i$ and membrane voltage in single $Me_2BAPTA$-loaded cells stimulated with 1 mM carbachol in $Ca^{2+}$ medium. (*A*) In some cells, $[Ca^{2+}]_i$ began to rise ≈300–1500 msec after the initial hyperpolarization. (*B*) In other cells, $[Ca^{2+}]_i$ remained unchanged during the first several seconds following the initial hyperpolarization. ○, Membrane voltage; ●, $[Ca^{2+}]_i$.

## DISCUSSION

Two possible explanations were given for the observation that the earliest detectable membrane hyperpolarization following carbachol stimulation of rat parotid acinar cells precedes the earliest detectable rise in $[Ca^{2+}]_i$. The first, that $[Ca^{2+}]_i$ is not the relevant intracellular messenger, appears highly unlikely. This is supported by (*i*) the parallel diminutions of the rise of $[Ca^{2+}]_i$ and membrane hyperpolarization during repeat stimulation of normal cells in zero-$Ca^{2+}$ medium (Fig. 1), (*ii*) the elimination of both responses in $Me_2BAPTA$-loaded cells stimulated in zero-$Ca^{2+}$ medium (Fig. 2*A*) in contrast to the ability of $Me_2BAPTA$-loaded cells to hyperpolarize in $Ca^{2+}$ medium (Fig. 3), and (*iii*) the ability of the $Ca^{2+}$ ionophore ionomycin to cause a rapid membrane hyperpolarization similar to that observed with carbachol (data not shown). Taken together with results from patch-clamp studies that indicate that $Ca^{2+}$-activated $K^+$ channels underlie the membrane hyperpolarization observed in response to carbachol (1), these data provide strong evidence that a rise of $[Ca^{2+}]_i$ is directly responsible for the activation of these channels and the associated membrane hyperpolarization.

The second explanation for the temporal relationship between the hyperpolarization and the rise of $[Ca^{2+}]_i$ is that $[Ca^{2+}]$ rises at the membrane before it rises in the bulk cytoplasm. Implicit in this explanation is the conclusion that fura-2 is unable to detect a highly localized, fast change of $[Ca^{2+}]$ at the basolateral membrane in the vicinity of the $Ca^{2+}$-activated $K^+$ channels. This conclusion is supported by experiments in which $Me_2BAPTA$-loaded cells were stimulated in $Ca^{2+}$ medium. These experiments were expressly designed to produce a transient, local rise of $Ca^{2+}$ at the plasma membrane followed by a more gradual increase in $[Ca^{2+}]_i$ in the cytoplasm as $Ca^{2+}$ influx from the extracellular solution saturated intracellular $Me_2BAPTA$. In these studies, carbachol produced a hyperpolarization that considerably preceded any fura-2 detectable increase of $[Ca^{2+}]_i$. The magnitude of the hyperpolarization was equivalent to that observed in unloaded cells, indicating that $[Ca^{2+}]$ at the inner surface of the basolateral membrane rose sufficiently to activate the $K^+$ channels to the extent observed in control cells. However, in most cells the rate of hyperpolarization was considerably slowed (compare Fig. 3 *A* and *B* with Fig. 1*A*), which is as expected since $Ca^{2+}$ entering

the cell by way of the basolateral membrane must first interact with a large pool of $Ca^{2+}$ chelator ($Me_2BAPTA$) at the inner surface of the membrane. The observed slow kinetics of cytosolic $[Ca^{2+}]_i$ measured with fura-2 are likewise consistent with $Ca^{2+}$ interacting with a large $Ca^{2+}$-buffer pool throughout the cytoplasm. Cell-to-cell variability in the $Me_2BAPTA$-loading efficiency, as well as in the magnitude of the agonist-induced $Ca^{2+}$ influx, would be expected to generate variability in the kinetics of hyperpolarization and $[Ca^{2+}]_i$ among these cells, as observed.

These experiments clearly demonstrate that, when $[Ca^{2+}]$ is expected to rise first at the plasma membrane, the membrane can indeed sense that $Ca^{2+}$ before it can be detected in the cytoplasm. Taken together with previous results (6, 18–28), the data presented here indicate that the magnitude and kinetics of the voltage response to carbachol provide an independent measure of $[Ca^{2+}]$ at the inner surface of the basolateral membrane. Thus, the most likely explanation for the observation that the rise of fura-2-detected $[Ca^{2+}]_i$ does not precede the membrane hyperpolarization in normal cells stimulated in zero-$Ca^{2+}$ medium (Fig. 1*D*) is that the earliest rise of $[Ca^{2+}]_i$ is localized near the plasma membrane. Since the only source of mobilized $Ca^{2+}$ in zero-$Ca^{2+}$ medium is intracellular stores, those stores must be localized at or near the basolateral membrane. By a similar argument, $Ca^{2+}$ from the extracellular medium must also enter the cytoplasm at or close to the basolateral membrane.

The experiments shown in Fig. 2 provide further evidence that the agonist-sensitive intracellular pool of $Ca^{2+}$ is localized near the basolateral membrane. Although $Me_2BAPTA$-loaded cells stimulated in zero-$Ca^{2+}$ medium fail to hyperpolarize or show a rise of $[Ca^{2+}]_i$, after these cells were exposed for a brief (3 min) period to $Ca^{2+}$ medium following a stimulation in zero-$Ca^{2+}$ medium (Fig. 2*B*), a repeat stimulation in zero-$Ca^{2+}$ medium elicited a normal, rapid hyperpolarization (Fig. 2*C*), which was not associated with a fura-2-detectable increase of $[Ca^{2+}]_i$ (data not shown). The most plausible explanation for these data is that $Me_2BAPTA$ depletes the agonist-sensitive intracellular stores of $Ca^{2+}$ and that exposure to extracellular $Ca^{2+}$ following stimulation replenishes them. Subsequent stimulation causes a release of $Ca^{2+}$ from these stores, which is sensed by the basolateral membrane, causing it to hyperpolarize (as for non-BAPTA-loaded cells). Since this released $Ca^{2+}$ is not detected by fura-2, the $Ca^{2+}$ released from the stores must be localized in the immediate vicinity of the basolateral membrane. The amount of $Ca^{2+}$ released is sufficient to saturate the $Me_2BAPTA$ adjacent to the membrane but insufficient to overcome the buffering in the cytoplasm, which allows $[Ca^{2+}]$ to rise only at the basolateral membrane and cause a normal hyperpolarization.

In rat parotid acinar cells (9, 12, 13), as in many other cell types (35), $Ca^{2+}$ is mobilized from intracellular stores by inositol trisphosphate. Inositol trisphosphate is formed at the basolateral membrane by receptor–ligand activation of phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate (9, 12, 36–40). In many cell types (41), including the parotid acinar cell (13, 42), inositol trisphosphate-induced $Ca^{2+}$ release is associated with a microsomal fraction enriched in endoplasmic reticulum markers. Whether the entire endoplasmic reticulum, only a specialized fraction of it (35, 42–47), or another organelle (48) represents the $Ca^{2+}$ pool is unresolved. Although the endoplasmic reticulum is distributed throughout the cytoplasm of the parotid acinar cell, as in most cells, the results of the present study demonstrate that a receptor-mobilized pool of $Ca^{2+}$ must be localized at or near the basolateral membrane. Other studies are now required to define the physical basis of this pool. A recent model by Putney (49) also proposes a close physical association between the plasma membrane and the agonist-sensitive intracellular pool of $Ca^{2+}$ in order to explain refilling of that pool following stimulation. Subcellular localization of $Ca^{2+}$ pools may represent a general mechanism employed by cells to promote agonist-induced increases in $[Ca^{2+}]$ at spatially discrete intracellular sites.

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