# Flash photolysis studies of the localization of calcium release sites in rat parotid isolated acinar cells

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1. The temporal relationship between cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and activation of membrane current responses in single rat parotid acinar cells has been examined. Activation of muscarinic receptors by carbachol (CCh) at −40 mV (midway between $E_K$ and $E_{Cl}$ under our experimental conditions) frequently evoked biphasic current responses, application of 2 $\mu$M CCh leading to rapid activation of an inward current followed by a slower outward current.

2. Photochemical release of inositol 1,4,5-trisphosphate ($InsP_3$), from 'caged' $InsP_3$, by a brief near-UV flash, evoked similar biphasic current responses at −40 mV. In contrast, elevation of $[Ca^{2+}]_i$ by photolysis of the caged calcium compound nitr-5 at −40 mV activated only monophasic current responses.

3. These results can be explained by a model in which the $InsP_3$-sensitive $Ca^{2+}$ release sites are localized at the luminal pole of the cell, combined with a relative preponderance of $Ca^{2+}$-activated $Cl^-$ channels at that pole, and a relative preponderance of $Ca^{2+}$-activated $K^+$ channels at the basal end.

Cytosolic free $Ca^{2+}$ ions play an essential physiological role in a wide range of cell functions; for example, triggering of exocytosis in excitable or non-excitable cells requires elevation of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_i$), as does the secretion of electrolyte by exocrine tissue. In many non-excitable tissues, such as exocrine acinar cells, this $Ca^{2+}$ signal is mediated by inositol 1,4,5-trisphosphate ($InsP_3$) which evokes release of $Ca^{2+}$ from intracellular stores. These stores are believed to be a part of the rough endoplasmic reticulum (ER), or a specific $Ca^{2+}$ storage organelle (Streb, Bayerdorffer, Haase, Irvine & Schulz, 1984; Volpe *et al.* 1988).

The localization of these stores, and of the calcium release sites, may have significance for the mechanisms by which cells fulfil their physiological roles. In addition, physiological evidence concerning the localization of the stores will assist in determining the nature of the anatomical features that form the stores. Conflicting reports have been published concerning the localization of $Ca^{2+}$ stores and release sites in exocrine acinar cells. In rat parotid acinar cells Foskett, Gunter-Smith, Melvin & Turner (1989) suggested that the receptor-mediated release sites were localized near the basolateral membrane. In contrast, Kasai & Augustine (1990) reported that in rat pancreatic acinar cells they were localized at the apical end of the cell. More recently, $Ca^{2+}$ release has been shown to occur at the apical pole of mouse pancreatic and lacrimal acinar cells (Toescu, Lawrie, Petersen & Gallacher, 1992) and of rat lacrimal acinar cells (Tan, Marty & Trautmann, 1992). In rat parotid acinar cells, the elevation of $[Ca^{2+}]_i$ resulting from stimulation of muscarinic receptors induces both an outward current, carried mainly by $K^+$ ions through $Ca^{2+}$-activated $K^+$ channels, and an inward current, carried mainly through $Ca^{2+}$-activated $Cl^-$ channels (Iwatsuki, Maruyama, Matsumuto & Nishiyama, 1985; Gray, 1988*a*). In single isolated parotid acinar cells, held at a membrane potential of −40 mV, we have observed that muscarinic agonists evoke a biphasic electrical response of rapid net inward current followed by a slower, larger net outward current. The biphasic nature of these responses may arise from the differential localization of the relevent $Ca^{2+}$ release sites. In this work we have attempted to determine the localization within one cell of these $Ca^{2+}$ release sites.

The mechanism of the biphasic responses has been investigated by applying agents that activate the biochemical chain leading to $Ca^{2+}$ binding to ion channels at different points. Thus, the nature of the responses to $InsP_3$ and $Ca^{2+}$ applied directly into the cytosol has been examined and compared with those obtained by application of a muscarinic agonist. This has been achieved by loading cells with the photolabile agents 'caged' $InsP_3$ (Walker, Somlyo, Goldman, Somlyo & Trentham, 1987) and the caged calcium compound nitr-5 (Gurney, Tsien & Lester, 1987). The use of these agents allows rapid homogeneous release of $InsP_3$ and $Ca^{2+}$, respectively, into the cytosol, overcoming the problems of slow diffusion coupled with

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enyzmatic action and buffering, associated with application via the patch pipette.

Our results indicate that the Ins$P_3$-sensitive release sites in these cells are localized at the luminal pole. A preliminary report of some of this work has been made (Hassoni & Gray, 1992).

## METHODS

### Cell dissociation

Single, dissociated rat parotid acinar cells were prepared as described by Gray (1988*b*) with slight modifications. Briefly, male Wistar rats were killed by stunning and dislocation of the neck after which the parotid glands were isolated and washed with cold physiological saline. The composition of the saline solution used here, and as an external perfusate during experiments, was as follows (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; Hepes, 5 (pH 7·2).

Glands were finely minced and shaken at 37 °C for 15 min in $Ca^{2+}$- and $Mg^{2+}$-free Earle's balanced salts, (GIBCO) with 2 mM EDTA and 0·5% bovine serum albumin BSA (Sigma, UK) added. The tissue was resuspended in Minium Essential Medium with Earle's salts (GIBCO) including 0·15% collagenase, 0·15% hyaluronidase, 0·1% trypsin inhibitor and 1% BSA. It was then shaken at 37 °C for a further 40 min. During the period of enzyme treatment, the tissue was dispersed by trituration in a fine glass pipette at 20 and 40 min. Finally the tissue was resuspended and washed twice in saline before being plated on glass coverslips.

All recordings were made from cells within 3 h of dissociation. Cells viewed at this time still showed clear morphological polarity in that zymogen granules could be seen clustered at one pole of the cell. Similar results have been reported in mouse pancreatic acinar cells by Toescu *et al.* (1992).

### Whole-cell current recording

Recordings were made using the tight-seal whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were drawn from Pyrex glass (GC150TF-10, Clark Electromedical, Reading, UK). The patch pipette solution contained (mM): KCl, 150; $MgCl_2$, 2; ATP, 1; and Hepes, 5; and 200 $\mu$M EGTA, pH 7·2. When required, the photolabile compounds 'caged' Ins$P_3$ or nitr-5 (both supplied by Calbiochem, Novabiochem, Nottingham, UK) were loaded into cells by including them in the pipette solution. Transmembrane currents under voltage clamp were recorded using a List EPC-7 patch-clamp amplifier (List-electronic, Darmstadt/Eberstadt, Germany) and stored on videotape by Sony PCM701 digital audio processor, modified as described by Lamb (1985).

### $Ca^{2+}$ estimation

Estimation of $[Ca^{2+}]_i$ was made using the fluorescent dye fura-2 (Grynkiewicz, Poenie & Tsien 1985) as previously described (Gray, 1988*b*). In these experiments 200 $\mu$M fura-2 was added to the pipette solution, replacing the EGTA. The excitation wavelengths used were 360 and 380 nm; 360 nm was used as it is the isosbestic wavelength of fura-2 and thus provides a $[Ca^{2+}]_i$-independent record of intracellular fura-2 concentration. Calibration was carried out *in vitro* with droplets of patch pipette solution containing fura-2 and calcium, buffered to a range of concentrations with EGTA. No allowance for the effects of cytoplasmic viscosity was made. As viscosity and ionic strength are known to affect the dissociation constant of $Ca^{2+}$ from fura-2 (Williams & Fay, 1990) the calibrations obtained must be taken as estimates; for this reason we have calibrated the $y$-axis in Fig. 1*A* in both estimated $[Ca^{2+}]_i$ and fluorescence ratio. The calibrations obtained here did, however, agree well with those obtained previously in this laboratory (Gray, 1988*b*) *in situ* by loading single acinar cells with fura-2 and calcium buffers, and for this reason we do not expect the errors due to failure to allow for viscosity to be very large.

### Application of carbachol (CCh) and photolabile compounds

Carbachol (CCh) was applied through a continuous bath perfusion system. All the recordings were performed at room temperature (23–25 °C) unless otherwise stated. Solutions could be changed in about 3 s, consisting of a 2 s dead time and a 1 s exchange period. Photolysis of caged compounds was performed using a Model JML xenon arc flash lamp (Rapp & Guth, 1988). The light pulses (approximately 1 ms duration) were transmitted by a liquid light guide through the objective of the microscope.

## RESULTS

### Biphasic current responses to muscarinic agonist

Figure 1 shows a typical response from a isolated rat parotid acinar cells to the muscarinic agonist carbachol (CCh). $[Ca^{2+}]_i$ and whole-cell current were recorded simultaneously. The cell was voltage clamped at −40 mV, midway between $E_K$ and $E_{Cl}$, estimated as −85 mV and 0 mV, respectively. There was a rapid elevation of $[Ca^{2+}]_i$, to a peak of about 1400 nM, followed by decaying oscillations. The current followed these oscillations except during the first few seconds of the response, during which time there was a brief inward current peak. The net inward current rapidly disappeared, and was followed by a net outward current for the remainder of the response. The results suggested that the inward current was both more rapidly activated than the outward current, and also that it was smaller than the outward current at this holding potential, once the latter was fully activated.

Such early peaks of inward current where seen in a number of cells, though not in all. Figure 2*B* shows the current recording, on a faster time scale, from another cell in which a biphasic current response was obtained at −40 mV. In this case no measurement of $[Ca^{2+}]_i$ was made. In Fig. 2*C* a response is shown from a cell which showed a monophasic current response. A resolvable biphasic response was obtained in eight cells out of twelve with 2 $\mu$M CCh.

### Membrane currents evoked by photolysis of 'caged' Ins$P_3$

The variability of the appearance of biphasic responses could result from the relatively slow kinetics of the responses to CCh, which in turn results from the slow kinetics of the

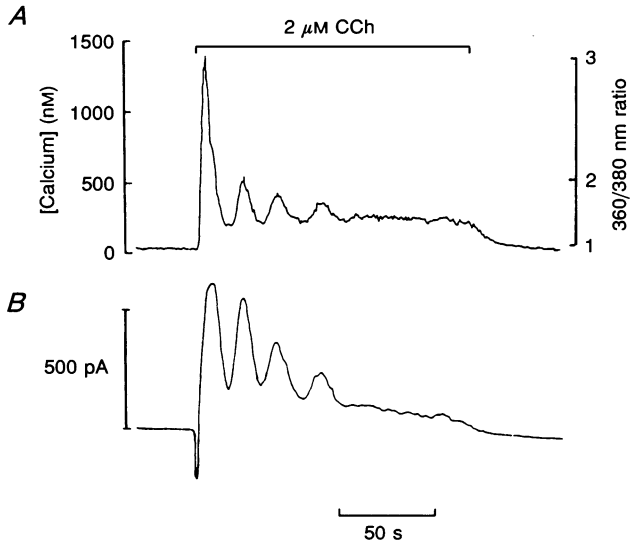


**Figure 1. $Ca^{2+}$ and current response to CCh**
Combined estimation of $[Ca^{2+}]_i$ using fura-2 free acid (*A*) and whole-cell current recording (*B*) at −40 mV. 2 μM CCh was applied by bath perfusion. *A*, the left-hand axis shows the estimated $[Ca^{2+}]_i$, the right-hand axis the measured ratio of fluorescence at 360/380 nm excitation. An initial biphasic current response is followed by outward current oscillations in phase with the $[Ca^{2+}]_i$ signal. Following wash-off of CCh, both signals decayed at the same rate. Biphasic current responses, such as this, were observed in 8 out of 12 cells challenged with 2 μM CCh.

muscarinic receptor and the bath perfusion system. In order to overcome this limitation, and to investigate further the mechanism of the responses, we stimulated the cells with $InsP_3$, which was applied rapidly into the cytosol by flash photolysis of 'caged' $InsP_3$. Figure 2*B* shows the results of such an application. A greatly pronounced initial inward current is seen. These currents arose because the free $InsP_3$ which was liberated by photolysis induced $Ca^{2+}$ release from intracellular stores. This result is typical; such responses were seen in five out of five cells loaded with 10 μM 'caged' $InsP_3$, seven out of eight cells at 20 μM caged $InsP_3$ and three out of three cells at 50 μM caged $InsP_3$.

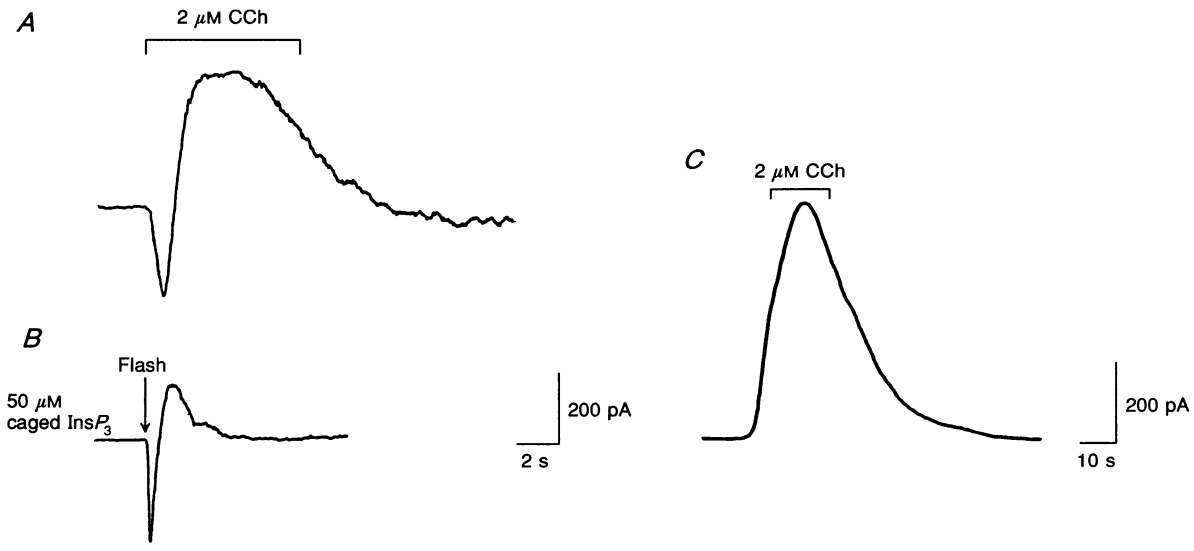


**Figure 2. Current responses to CCh and $InsP_3$**
Current responses of three cells voltage clamped at −40 mV. *A*, biphasic current response obtained in response to bath application of 2 μM CCh. Biphasic responses were obtained in 8 cells out of 12 challenged with 2 μM CCh. *B*, a biphasic response to photolysis of 50 μM caged $InsP_3$, loaded into the cell by inclusion in the patch pipette solution. Biphasic responses were obtained in 15 cells out of 16 challenged by flash photolysis of 'caged' $InsP_3$. *C*, a monophasic current response obtained in response to bath application of 2 μM CCh; such monophasic responses were obtained in 4 out of 12 cells challenged with 2 μM CCh.

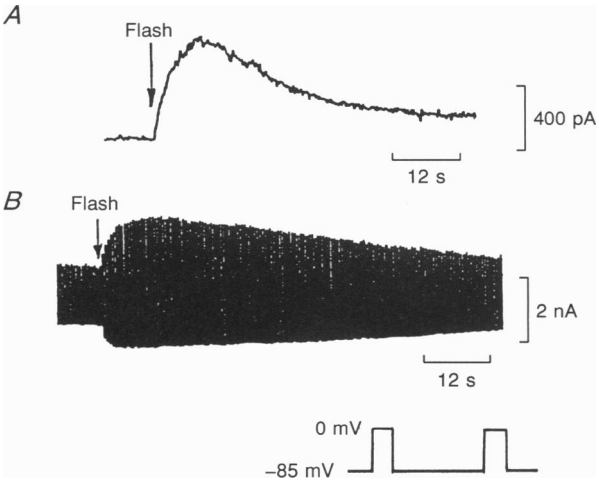


**Figure 3. Current responses to $Ca^{2+}$**
Responses to photolysis of a nitr-5-loaded cell. The cell was loaded with 2 mM nitr-5 (buffered to an initial free $Ca^{2+}$ of 70 nM), which was included in the patch pipette solution. *A*, cell voltage clamped at −40 mV. *B*, cell voltage clamped at −85 mV and stepped to 0 mV following the protocol illustrated. All ten cells challenged by flash photolysis of 2 mM nitr-5 gave monophasic responses, as shown here.

As photolysis of caged $InsP_3$ should release $InsP_3$ rapidly, within about 1 ms (Walker *et al.* 1987), and occur homogeneously throughout the cell, the observed biphasic responses could result from localization of the $InsP_3$-mediated $Ca^{2+}$ release sites, coupled with localization of the different ion channel types, or it could result from differences in the kinetics of the channels themselves, such as in the binding of $Ca^{2+}$.

## Membrane currents evoked by photolysis of nitr-5

To choose between the two possible models we stimulated the cells by rapid release of $Ca^{2+}$ from the photolabile $Ca^{2+}$ buffer nitr-5, as this should give rise to a rapid, homogeneous elevation of $[Ca^{2+}]_i$ throughout the cell. In this case we only ever observed monophasic responses to release of $Ca^{2+}$ initiated by a flash. Figure 3*A* shows such a response from a cell loaded with 2 mM nitr-5 buffered to give an initial free $[Ca^{2+}]_i$ of 70 nM.

The lack of a biphasic response could result from the evoked elevation of $[Ca^{2+}]_i$ being insufficient to activate the inward conductance, which is known to be less sensitive to $Ca^{2+}$ than the $K^+$ conductance (Marty, Tan & Trautmann, 1984). To eliminate this possibility, further experiments were carried out in which the holding potential was stepped between $E_K$ and $E_{Cl}$ during the response. Figure 3*B* shows such a response. Both inward and outward currents are activated by the released $Ca^{2+}$, indicating that the lack of a biphasic response results from simultaneous activation of the two conductances, rather than the activation of a single conductance.

This concentration of nitr-5 (2 mM) would also effectively buffer $[Ca^{2+}]_i$; in three cells loaded with 2 mM nitr-5, no responses were obtained following the application of CCh (2–5 μM). Thus, a strong component of localized $Ca^{2+}$-induced $Ca^{2+}$ release could be 'hidden' when the cells are loaded with 2 mM nitr-5. For this reason we examined the responses to a flash of cells loaded with a range of nitr-5 concentrations, all prebuffered with $Ca^{2+}$ to give an initial free $Ca^{2+}$ of 70 nM. Figure 4 shows a result obtained with 200 μM nitr-5. The response is similar to that seen with 2 mM nitr-5, though much smaller in amplitude. With 200 μM nitr-5, visible responses were only obtained in three out of five cases; in none of these cases was a biphasic response seen. At these levels of nitr-5 little interference with the normal $Ca^{2+}$ release and buffering mechanisms of the cell is expected.

## DISCUSSION

Our results demonstrate biphasic current responses from single rat parotid acinar cells voltage clamped at −40 mV. These occur when stimulated by muscarinic agonists, which activate current via an elevation of $[Ca^{2+}]_i$ mediated by the action of $InsP_3$. The rapid activation of inward current could result from a variety of mechanisms. Firstly, kinetics of the $Cl^-$ conductance could activate more rapidly; though this seems unlikely as this is known to be less sensitive to

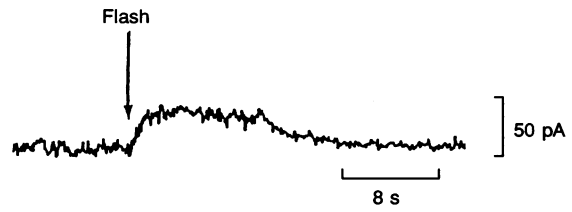


**Figure 4. Current response with low nitr-5 concentration**
Response of a cell loaded with 200 μM nitr-5 (buffered to an initial free $[Ca^{2+}]$ of 70 nM), which was included in the patch pipette solution. The cell was voltage clamped at −40 mV. Out of 5 cells challenged by flash photolysis of 200 μM nitr-5, 3 gave monophasic responses, such as shown here and 2 gave no resolvable response.

$[Ca^{2+}]_i$ than the outward, $K^+$ current (Marty *et al.* 1984; Evans & Marty, 1986). Secondly, the receptor for the agonist, or the sites of production of $InsP_3$, might be localized near a region in which there is a relative preponderance of the channels carrying the inward current, while $InsP_3$-sensitive $Ca^{2+}$ release sites are distributed evenly through the cell. Thirdly, the release sites themselves might be localized near to the channels. Fourthly, localized elevation of $[Ca^{2+}]_i$ near to the channels carrying the inward current could occur if $Ca^{2+}$-activated $Ca^{2+}$ release played a major role in the elevation of $[Ca^{2+}]_i$ and if the mechanisms for $Ca^{2+}$-activated release were localized near to the channels responsible for carrying the inward current.

The observation that the currents obtained with flash photolysis of caged $InsP_3$ are more clearly biphasic than those obtained with agonist (CCh) discounts localization of the receptors or $InsP_3$ production as a possibility, as under these experimental conditions $InsP_3$ is released evenly through the cell. The possibility that the channels carrying inward current activate more rapidly than the $K^+$ channels is ruled out by the results obtained with nitr-5. When a homogeneous elevation of $[Ca^{2+}]_i$ is generated biphasic currents are not seen; both conductances activate with essentially similar time courses. The choice between the last two options is more difficult. However, previous work (Gray, 1988*b*) has indicated that $Ca^{2+}$-activated $Ca^{2+}$ release is unlikely to play a significant role in the generation of the oscillations of $[Ca^{2+}]_i$ seen in these cells. In addition, when low concentrations (200 $\mu$M) of nitr-5 are used, to ensure that the nitr-5 does not play a significant role in buffering $[Ca^{2+}]_i$ elevation, monophasic responses are still seen (Fig. 4). Under these conditions the elevation of $[Ca^{2+}]_i$ evoked by a flash is small, but if $Ca^{2+}$-activated $Ca^{2+}$ release played a major role in determining the distribution of $[Ca^{2+}]_i$ elevation in the cell, some triggering of localized release might be expected. This observation contrasts with reports from rat lacrimal acinar cells (Marty & Tan, 1989) and from mouse pancreatic acinar cells (Wakui, Osipchuk & Petersen, 1990) in which evidence for a significant component of calcium-induced calcium release was presented. This contrast may reflect either that the pulse of $Ca^{2+}$ released by flash photolysis of 200 $\mu$M nitr-5 being too small or too slow to activate calcium-induced calcium release, or calcium-induced release occurring but not being localized to one pole of the cell. On balance, we believe the most likely explanation to be that both the channels carrying the inward current and the $InsP_3$-sensitive $Ca^{2+}$ release site are localized within the same region of the cell.

One additional possibility would be that the localized elevation of $Ca^{2+}$ arose from rapid entry through localized $Ca^{2+}$ entry sites, rather than localized release sites. However, such a model can be ruled out as it is clear that only a very small proportion of the initial $Ca^{2+}$ elevation that results from muscarinic stimulation of rat parotid acinar cells comes from outside the cell (Merritt & Rink, 1987). Biphasic responses, as reported here, could only arise if a major fraction of the $Ca^{2+}$ entering the cytosol came from localized entry sites.

The region where the $Ca^{2+}$ release sites and inward conductance are localized must be the luminal membrane of the cell for a number of reasons. Firstly, it is well known that the $Ca^{2+}$-activated $K^+$ channels are present in large numbers on the basolateral surface of the cell (Petersen & Maruyama, 1984); the $Cl^-$ channels must be localized elsewhere to explain the biphasic responses. Secondly, work in this laboratory has demonstrated that isolated patches from apical membranes of these cells contain a significantly higher density of $Cl^-$-activated channels than those from the basolateral membrane (C. Magnus, unpublished observations). Thirdly, this latter observation is supported by reports from both rat pancreatic acinar cells (Kasai & Augustine, 1990) and rat lacrimal acinar cells (Tan *et al.* 1992) in which $Ca^{2+}$ imaging was used to show that release of $Ca^{2+}$ occurs initially at the apical end of the cell, and that it is associated with an initial $Cl^-$ current. We thus conclude that the biphasic responses result from localization of $InsP_3$-sensitive $Ca^{2+}$ release sites at the apical end of the cell, combined with a preferential localization of $Cl^-$ channels at the same end of the cell. Our results do not allow any conclusions to be drawn as to whether $Cl^-$ channels are exclusively localized at the apical end, or simply preferentially localized. They do, however, require a similar preferential localization of the $Ca^{2+}$ activated $K^+$ channels on the basolateral membrane.

This conclusion contrasts with that of Foskett *et al.* (1989) who demonstrated in isolated rat parotid cells stimulated with a muscarinic agonist that a hyperpolarization, recorded with an intracellular microelectrode, preceded the observed elevation of mean $[Ca^{2+}]_i$ by several milliseconds. The authors concluded that the $Ca^{2+}$ release sites were localized near the basolateral membrane, as the $K^+$ conductance, which was believed to be responsible for the hyperpolarization, is localized there. These observations are not inconsistent with ours, though the conclusions are. *In vivo*, $E_{Cl}$ is about $-30$ mV. The only membrane potential value quoted by Foskett *et al.* (1989) is $-28$ mV. At this potential, activation of the $Cl^-$ conductance will produce either no effect or a small hyperpolarization. Activation of the $K^+$ conductance will also evoke a hyperpolarization. If a small proportion of the $Ca^{2+}$-activated $K^+$ channels are situated near the luminal end of the cell then a marked hyperpolarization could easily be observed to precede elevation of mean $[Ca^{2+}]_i$, under the conditions used. Under our experimental conditions, such a component of early activating $K^+$ conductance would be swamped by the $Cl^-$ current, giving rise to the observed initial net inward current. Our observations are also consistent with single-

electrode voltage recordings made on mouse parotid acinar cells by Gallacher & Petersen (1980), who observed biphasic current responses resulting from release of ACh from nerve terminals in response to isolated field stimuli applied to slices of parotid gland. These authors recorded an initial depolarization with a duration of about 1 s followed by a hyperpolarization, from cells with a resting potential of about −65 mV.

Petersen (Petersen & Maruyama, 1984; Petersen, 1992) has proposed that there is a transmembrane cotransport system in the basolateral membrane of the parotid cell. The system uses a driving force generated by the activity of the $Na^+$ pump to allow the cycling of $K^+$ and the net influx of $Cl^-$ across the basolateral membrane. The model requires $Cl^-$ channels on the luminal membrane, which allow $Cl^-$ to diffuse passively into the lumen. The physiological significance, if any, of the early activation of $Cl^-$ conductance by agonist is not clear at present. In pancreatic acinar cells Kasai & Augustine (1990) proposed an alternative push–pull model of secretion, in which an early phase of hyperpolarization resulted from the early $Cl^-$ channel activation, as the resting potential is somewhat positive to $E_{Cl}$. This would be followed by a depolarization as the $Ca^{2+}$ wave reached the basolateral membrane, where $Ca^{2+}$ activated non-selective cation channels are situated, in rat pancreatic acinar cells. The alternation of hyper- and depolarization is proposed to allow passive exit of $Cl^-$ into the lumen, followed by passive entry via the basal membrane. However, such a model cannot operate in parotid acinar cells as in these cells the predominant channels on the basolateral membrane are $Ca^{2+}$-activated $K^+$ channels, which hyperpolarize the cell on activation.

The localization of release of $Ca^{2+}$ does, however, have implications for exocytosis in these cells. During maintained release $Ca^{2+}$ will diffuse throughout the cell, and activate $Ca^{2+}$-sensitive processes in all parts. However, if stimulation of the cell occurs as a series of transients, then $Ca^{2+}$ will be preferentially elevated at the apical end of the cell. Most notably the effect of this is likely to be a preferential activation of exocytosis, which is known to be $Ca^{2+}$ sensitive in these cells (Baldys-Waligorska, Pour, Moriarty & Dowd, 1987). Thus the pattern of stimulation of the cells may provide a method of control of the relative proportions of protein to fluid and electrolyte secreted. The pattern of stimulation *in vivo* is unclear. Responses of mouse parotid acinar cells to ACh released from nerve terminals in response to field stimulation show discrete transients at 1 Hz that merge into a steady response above 5 Hz (Gallacher & Petersen, 1980). Thus, at lower frequencies such relative localization of mean $[Ca^{2+}]_i$ could occur. Local oscillations of $[Ca^{2+}]_i$ would tend to preferentially activate exocytosis. Such oscillations have been observed in these cells (Gray, 1988*b*), though it is unclear whether they occur *in vivo*. In conclusion, stimulation of rat parotid acinar cells by muscarinic agonists leads to an initial rapid activation of $Cl^-$ conductance, which precedes the activation of $K^+$ conductance. This results from the localization of the $InsP_3$- sensitive $Ca^{2+}$ release sites at the luminal pole of the cell; combined with a relatively higher density of $Cl^-$ channels on the luminal membrane and of $K^+$ channels on the basolateral membrane.

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