Evidence that Ca^{2+} cycling by the plasma membrane Ca^{2+}-ATPase increases the 'excitability' of the extracellular Ca^{2+}-sensing receptor

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Summary

The extracellular Ca^{2+}-sensing receptor (CaR) is a widely expressed G-protein-coupled receptor that translates information about $[Ca^{2+}]$ in the extracellular milieu to the interior of the cell, usually via intracellular Ca^{2+} signaling pathways. Using fura-2 imaging of cytoplasmic $[Ca^{2+}]$, we observed that HEK293 cells expressing CaR oscillated readily under conditions permissive for CaR activation. Spiking was also triggered in the absence of external Ca^{2+} by the CaR agonist spermine (1 mM). Oscillating cells were typically located in clusters of closely apposed cells, but Ca^{2+} spiking was insensitive to the gap junction inhibitor 18α-glycyrrhetinic acid. We hypothesized that Ca^{2+} signals might be amplified, in part, through a positive feedback loop in which Ca^{2+} extrusion via the plasma membrane Ca^{2+}-ATPase (PMCA) activates CaRs on the same cell or adjacent cells through local increases in $[Ca^{2+}]_{out}$. In support of this idea, addition of exogenous Ca^{2+} buffers (keeping free $[Ca^{2+}]_{out}$ constant) attenuated or eliminated Ca^{2+} signals (manifested as oscillations), as did PMCA inhibitors ($HgCl_2$, orthovanadate and Caloxin 2A1). Measurement of extracellular $[Ca^{2+}]$ using the near membrane probe fura-C_{18} revealed that external $[Ca^{2+}]$ rose following receptor activation, sometimes displaying an oscillatory pattern. Our data suggest that PMCA-mediated cycling of Ca^{2+} across the plasma membrane leads to localized increases in $[Ca^{2+}]_{out}$ that increase the excitability of CaR.

Key words: Calcium oscillations, Extracellular signals, Intercellular communication

Introduction

The extracellular Ca^{2+}-sensing receptor (CaR) is a cell surface receptor for divalent cations whose existence was originally suspected in the parathyroid gland (Brown et al., 1987; Nemeth and Scarpa, 1987; Shoback et al., 1988). Cloning of the receptor by Brown and colleagues (Brown et al., 1993) confirmed the essential role of CaR as a Ca^{2+} sensor in the parathyroid, and also set the stage for the identification of the receptor in a wide variety of tissues not involved in systemic Ca^{2+} homeostasis (Brown et al., 1998). This seven-transmembrane-spanning receptor couples, via heterotrimeric G-proteins, to several different signaling cascades, including the phospholipase C (PLC)/phosphoinositide/Ca^{2+} pathway (Brown and MacLeod, 2001). The binding of Ca^{2+} to the human parathyroid receptor is known to be a highly cooperative process, making CaR a good Ca^{2+} 'increment detector'. Although half-maximal activation of the receptor occurs around 3.5 mM, with maximal stimulation at 5.5 mM (Conigrave et al., 2000a; Quinn et al., 1997), CaR is able to sense small $[Ca^{2+}]$ changes (±0.2 mM) in the physiological range (around 1.8 mM Ca^{2+}).

It is important to note that CaR is also stimulated by Mg^{2+} and certain polycations [including some endogenous polyamines such as spermine and spermidine (Quinn et al., 1997)]. Synthetic small molecule agonists of the receptor, such as the allosteric modulator NPS R-467 (Nemeth et al., 1998), have also been prepared. Brown and colleagues recently showed that the CaR is activated by physiological concentrations of amino acids [particularly aromatic and small aliphatic L-amino acids (Conigrave et al., 2000b)], and that it is modulated by ionic strength (Quinn et al., 1998). It is therefore likely that multiple agonists act in concert to stimulate the receptor physiologically.

We noted previously that stimulation of CaR with maximal doses of CaR agonists was frequently manifested by an oscillatory Ca^{2+} signal in HEK293 cells transfected with the human extracellular Ca^{2+}-sensing receptor (HEK CaR cells) (Hofer et al., 2000). The same observation was made in CaR-transfected HEK cells by Breitwieser and Gama (Breitwieser and Gama, 2001), who also reported that Ca^{2+} spiking was acutely sensitive to small incremental increases in extracellular $[Ca^{2+}]$. Such oscillations were sustained for up to 40 minutes, until they gradually diminished in amplitude. Young and Rozengurt recently extended these findings, showing that aromatic amino acids can sensitize the receptor to small incremental increases in external $[Ca^{2+}]$ in HEK CaR cells, producing distinctive patterns of oscillations (Young and Rozengurt, 2002). Native tissues that express CaR endogenously, including parathyroid cells (Miki et al., 1995), thyroid follicular cells (McGehee et al., 1997) and pancreatic

acinar cells (Bruce et al., 1999), have also been observed to oscillate following stimulation with maximal doses of CaR activators, hinting that this mode of signaling may be a fundamental characteristic of the receptor.

The mechanisms underlying Ca^{2+} oscillations have been the subject of intense investigation ever since the first direct observations of this phenomenon were made by Cuthbertson and Cobbold in aequorin-loaded mouse oocytes (Cuthbertson and Cobbold, 1985). Numerous models have been put forth to account for Ca^{2+} oscillations (Berridge et al., 2000; Bootman et al., 2001; Thomas et al., 1996; Thorn et al., 1993). Prominent among these are themes involving sensitization of intracellular Ca^{2+} release channels by Ca^{2+} ions (Patel et al., 1999; Yule, 2001). Certain inositol (1,4,5)-trisphosphate ($InsP_3$) receptor subtypes are in fact known to have a bell-shaped dependency of open probability on cytoplasmic [Ca^{2+}], leading to alternate activation and inhibition of Ca^{2+} release as cellular [Ca^{2+}] rises (Bezprozvanny et al., 1991; Mak et al., 1998; Moraru et al., 1999). Oscillations in internal store [Ca^{2+}] have been observed directly in permeabilized cells, indicating a minimal requirement for intact functional stores and $InsP_3$ receptors (Hajnoczky and Thomas, 1997). These and other data (Miyakawa et al., 2001) would suggest that oscillations are an intrinsic property of $InsP_3$-sensitive internal Ca^{2+} stores.

Other mechanisms for Ca^{2+} oscillations have been described that rely on negative feedback exerted on PLC by PKC (Bird et al., 1993; Taylor and Thorn, 2001). Following activation of G-protein-coupled cell surface receptors, PLC hydrolyzes phosphatidylinositol (4,5)-bisphosphate (PIP_2) into DAG and $InsP_3$. DAG activates PKC, which, according to several models, exerts negative effects on PLC, resulting in oscillatory $InsP_3$ production. Confirmatory evidence for this model has been provided recently by experiments in which PIP_2 hydrolysis was measured in real-time in single living cells, using genetically encoded GFP-based fluorescent sensors. Meyer and colleagues (Codazzi et al., 2001) and Ferguson and colleagues (Dale et al., 2001) recently showed that $InsP_3$ levels in the cell, as well as PKC translocation to the plasma membrane do indeed oscillate, and with a time course that closely parallels that of Ca^{2+} spiking. These experiments confirm that $InsP_3$ is produced cyclically and may indicate that negative feedback loops involving PKC contribute to shaping this particular type of oscillatory behavior. Using similar techniques, Nash et al., also showed that stimulation of metabotropic glutamate receptor subtype 5a (mGluR5a) resulted in Ca^{2+} oscillations concurrent with oscillations in intracellular [$InsP_3$] (Nash et al., 2001b). However, oscillations in [$InsP_3$] were not noted when the same cells were stimulated with a different Ca^{2+} mobilizing agonist, methacholine, acting through muscarinic M_3 receptors, even though oscillations in intracellular [Ca^{2+}] were observed under these conditions. Thus it appears that different mechanisms for generating Ca^{2+} oscillations can co-exist even in the same cell.

We previously reported that heterotypic assemblages of cells may communicate with each other through extracellular [Ca^{2+}] increases that result from the extrusion of cellular Ca^{2+} across the plasma membrane during Ca^{2+} signaling events (Hofer et al., 2000; Thomas, 2000). These external [Ca^{2+}] increases are detected by CaR on adjacent cells, activating signaling cascades (including intracellular Ca^{2+} signals) in those cells. Moreover, using Ca^{2+}-selective microelectrodes, we recently performed direct measurements of extracellular [Ca^{2+}] changes in external microdomains of intact polarized gastric epithelium following stimulation with a Ca^{2+}-mobilizing agonist (Caroppo et al., 2001). We found that substantial increases in external [Ca^{2+}] (up to 0.5 mM) occurred at the apical face of the tissue in restricted microdomains near the membrane. Our data indicated that these changes were a consequence of PMCA-mediated Ca^{2+} extrusion from stimulated cells. These findings open up the possibility that the PMCA may exert feedback effects on CaR located on the same or adjacent cells.

In the present study we provide evidence for a novel autocrine mechanism that contributes to sustained Ca^{2+} signaling in CaR-expressing HEK-293 cells. Our data indicate that Ca^{2+} extruded to the extracellular space by the plasma membrane Ca^{2+} ATPase (PMCA) following intracellular Ca^{2+} spikes exerts a positive feedback effect on CaR by increasing local external [Ca^{2+}]. This additional stimulus enhances the likelihood of generating subsequent spikes in Ca^{2+}. Although it is probable that other 'classical' mechanisms are responsible for the genesis of Ca^{2+} oscillations in these cells, our data would suggest that cycling of Ca^{2+} across the plasma membrane following an intracellular Ca^{2+} transient reinforces CaR stimulation, and may modulate the periodicity of rhythmic spiking.

Materials and Methods

Cell culture

Human embryonic kidney cells stably transfected with the human CaR (Nemeth et al., 1998) and wild-type HEK293 cells were cultured in DMEM + Glutamax (Life Technologies, Grand Island, NY). Medium was supplemented with 10% fetal bovine serum, 10 units/ml penicillin and 10 μg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator (95% O_2, 5% CO_2). For cholesterol depletion experiments cells were incubated in the presence of 10 mM β-cyclodextrin in DMEM for 1 hour at 37°C. Transient transfection of HEK293 WT cells with human CaR (a generous gift of Mei Bai and Ed Brown (Harvard Medical School) was carried out using Quantum Prep Cytofectene Transfection reagent kit (Bio-Rad Laboratories, Hercules CA) according to the manufacturer's instructions.

Measurement of intracellular [Ca^{2+}]

CaR-transfected HEK cells were plated onto specially prepared glass coverslips as described previously (Hofer et al., 2000). Briefly, randomly arranged polypropylene fibers were melted onto the coverslip, resulting in the formation of small pockets between the fibers and the glass. Following plating, cells grew into these restricted spaces, and were thus partially shielded from the bulk solution. Only cells growing under such polypropylene pockets were selected for Ca^{2+} imaging experiments. Cells were cultured in this way for 48 hours and loaded with 2 μM fura-2 AM in DMEM for 40 minutes at 37°C. Coverslips were then mounted in an open-topped perfusion chamber (Series 20, Warner Instrument, Hamden CT) and placed on the stage of an inverted microscope (Olympus IMT-2). Test agents (diluted to final concentration in Ringer's) were often added directly to the chamber while the perfusion was stopped, resulting in complete exchange of the chamber volume (1 ml). In control experiments, addition of Ringer's solution alone to the chamber did not affect Ca^{2+} oscillations (not shown).

During the measurements cells were bathed with a Ringer's solution at 33°C containing 145 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.45 and $CaCl_2$ at the concentrations

indicated in the figures. Selected clusters of cells were excited alternately at 345 nm and 375 nm by a xenon arc lamp. Emitted fura-2 fluorescence at 510 nm was recorded every 5 seconds (frame averaging=16) by an intensified charge-coupled device camera (model IC-100; Photon Technology International, South Brunswick, NJ) and converted to pseudocolor images using ImageMaster 1.4 software (Photon Technology International). The data were expressed as the ratio of the fluorescence emitted at the two fura-2 excitation wavelengths (F_{340}/F_{380}). In a few imaging studies, fura-2 measurements were carried out using an alternate ratio imaging set-up running MetaFluor software (Universal Imaging, West Chester, PA). '*n*' refers to the number of independent experimental runs; data from 5 to 50 individual cells in the microscope field was collected and analyzed for each experiment. Statistical analyses were performed using InStat 2.03 (GraphPad software) using a paired *t*-test. Data are expressed as ±s.e.m., and differences considered highly significant when $P<0.005$; a *P*-value calculated by the program is provided for many of the data sets if very different from $P=0.005$. Representative tracings from single cells are shown. Calibrations were performed for some experiments according to the method of Grynkiewicz et al., assuming a K_d for fura-2 of 224 nM (Grynkiewicz et al., 1985).

Measurement of extracellular near-membrane [Ca^{2+}] with Fura-C_{18}-HEK CaR cells plated on glass coverslips without polypropylene mesh were incubated with 10 μM fura-C_{18} (Etter et al., 1994) in normal Ringer's for 3 minutes on the microscope stage and then rinsed in dye-free Ringer's. The fura-C_{18} ratio (F_{340}/F_{380}) was measured as described above for fura-2. The extracellular location of the dye was confirmed by quenching with 5 mM $NiCl_2$ in Ca^{2+}-free solution, which very rapidly reduced fluorescence intensity at both wavelengths to that of cellular autofluorescence (determined in the same cells prior to loading with dye). Treatment of fura-2-AM loaded cells with 5 mM $NiCl_2$ had no effect whatsoever on intracellular fura-2 fluorescence, confirming that Ni^{2+} is indeed impermeant to HEK293 cells.

Immunofluorescence staining

CaR-expressing HEK293 cells were washed in PBS (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and fixed in methanol for 10 minutes at –20°C. After rinsing in PBS at room temperature (25°C) cells were pretreated for 1 hour in PBS containing 1% BSA and 1% normal goat serum (used as a blocking solution to reduce nonspecific antibody binding). Samples were then co-incubated with a rabbit polyclonal anti-CaR antibody directed against amino acid residues 344-358 of the human CaR receptor diluted 1:100 in blocking solution, and with a mouse monoclonal anti-PMCA antibody, diluted 1:200 (Affinity BioReagents, Golden, CO), overnight at 4°C in a humidified chamber. Following five washes with 1% BSA in PBS, slides were incubated with goat anti-rabbit Alexa-Fluor-488-conjugated IgG (Molecular Probes, Eugene, OR) and goat anti-mouse affinity-purified Cy5-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), each diluted 1:500, for 1 hour at room temperature (25°C). After washing three times in PBS and once in distilled water, coverslips were mounted onto microscope slides with Pro-Long Antifade Kit (Molecular Probes, Eugene, OR). Controls for background and for nonspecific binding of the secondary antibody for PMCA were performed by omitting the anti-PMCA antibody. Controls for the nonspecific binding of the CaR antibody entailed co-incubation of samples with the CaR antibody and the immunizing peptide against which the antibody was made.

Confocal microscopy

Confocal fluorescence images (~1 μm sections) were obtained using a Bio-Rad (Hercules, CA) MRC1024ES multi-photon/confocal system with a krypton/argon ion laser excitation source. Cells were viewed with a Zeiss (Oberkochen, Germany) Axiovert S100 inverted microscope equipped with a high-quality c-apochromat water immersion objective (40×; 1.2 numerical aperture) in epifluorescence mode. The 512×512 pixel images were collected in a direct detection configuration at a pixel resolution of 0.484 μm with a Kalman 5 collection filter. Multiple labeled images were acquired in separate channels using narrow bandpass filters to restrict the emission wavelengths. Merged images of immunofluorescence staining were reconstructed using Metamorph software (Universal Imaging, West Chester, PA).

Confocal imaging of intercellular spaces

Cells were bathed in 10,000 M_r fluorescein-dextran (1 mg/ml in Ringer's solution), and a z-series (1 μm) of 25 to 40 confocal sections through a given cell cluster were acquired as described above using the 488 nm line of the krypton-argon laser (1% power; iris setting 1.4). The resulting stacks of images were processed and reconstructed into a side view profile (3 pixels or 1.452 μm in depth) using the 'kymograph' function of the Metamorph program.

Chemicals

NPS R-467 was a generous gift of E. Nemeth (NPS Pharmaceuticals, Toronto, Ontario, Canada). Fetal bovine serum was obtained from Life Technologies (Grand Island, NY). BAPTA tetrasodium salt, fura-2 acetoxymethyl ester, fura-C_{18} (pentapotassium salt), and fluorescein dextran (10,000 MW) were purchased from Molecular Probes (Eugene, OR). A stock solution of fura-C_{18} (1 mM) was prepared in Ringer's solution containing 1 mM $CaCl_2$. Caloxin 2A1 peptide (VSNSNWPSFPSSGGG-NH_2) was prepared by custom synthesis from Dalton Chemical Laboratories, Toronto ON, Canada. All other reagents were all purchased from Sigma (St Louis, MO).

Preparation of BAPTA-Ca^{2+} buffer- A buffered Ringer's solution was prepared containing 1 mM BAPTA free acid and 0.49 mM $CaCl_2$ to yield an estimated free [Ca^{2+}] of approximately 180 nM. Free [Ca^{2+}] was calculated with the MaxChelator program ('WEBMAXC v2.10'; http://www.stanford.edu/~cpatton/maxc.html). For the unbuffered solution, 10 μM BAPTA free acid was added to a nominally Ca^{2+}-free Ringer's solution (containing approximately 5 μM contaminating Ca^{2+}) to yield a calculated free [Ca^{2+}] of 175 nM. To confirm that the actual free [Ca^{2+}] was matched in each of the solutions, HEK293 cells loaded with fura-C_{18} were exposed sequentially to the two buffers during continuous measurement of the fura-C_{18} ratio. These measurements revealed that the free [Ca^{2+}] in the buffered solution (1 mM BAPTA/0.49 mM $CaCl_2$) was approximately 200 nM, whereas the unbuffered solution (10 μM BAPTA) had a lower ratio corresponding to a free [Ca^{2+}] that was approximately 150 nM. The fura-C_{18} ratio was converted into rough approximations of free [Ca^{2+}] using calibration techniques described previously (Etter et al., 1994).

Preparation of citrate-Ca^{2+} buffers

A modified Ringer's solution was prepared using 20 mM sodium citrate (NaCl substitution), pH 7.45. Ringer's solutions containing 1 mM or 0.65 mM added $CaCl_2$ were used as the control (unbuffered Ringer's) against which the free [Ca^{2+}] was matched in the buffered (+ citrate) solution. Free [Ca^{2+}] was matched precisely using a Ca^{2+}-selective electrode (model 97-20 ionplus, Orion Research, Beverly, MA) for each solution. The Ca^{2+}-buffering power of the citrate Ringer's solution is illustrated in Fig. 1E. Addition of $CaCl_2$ >600 μM was necessary to start detecting an increase in the measured free [Ca^{2+}] in the buffered solution. The Ca^{2+}-buffering power of the buffered Ringer's solution was at least twice as great as that of the control solution at 2 mM Ca^{2+}, since the free [Ca^{2+}] was elevated by less than half as much in the citrate-containing solution compared to control.

Results

Many cell types, including HEK293 cells, are known to oscillate when challenged with Ca^{2+} mobilizing agonists such as carbachol, particularly at sub-maximal doses (5-10 μM) (Luo et al., 2001; Shuttleworth, 1996). We and others had observed previously that HEK293 cells either transiently transfected or stably expressing CaR frequently displayed 'spontaneous' repetitive spikes in intracellular [Ca^{2+}] in physiological ambient [Ca^{2+}] (i.e. ~1.8-2.0 mM) (Breitwieser and Gama, 2001; Hofer et al., 2000). In the present study, we noted that conditions permissive for activation of the CaR, such as exposure to the allosteric CaR modulator NPS R-467 or the CaR agonist spermine, invariably gave rise to Ca^{2+} oscillations at a frequency of 1-2 spikes per minute. We further noted that vigorously oscillating cells tended to exist in clusters of closely apposed cells, while single cells growing in isolation rarely oscillated. However, oscillations were insensitive to an inhibitor of gap junctions, 18α-glycyrrhetinic acid (AGRA). Pretreatment of HEK CaR cells for 10 minutes with 50-100 μM AGRA did not inhibit the Ca^{2+} spiking induced by NPS R-467 (5 μM) or spermine (1 mM; not shown; *n*=3 experiments; 92 cells).

We considered the possibility that a component of the oscillatory phenomenon observed in the present study might result from a particular form of autocrine and/or paracrine signaling involving CaR and the PMCA. According to our hypothesis, Ca^{2+} spiking, once initiated, would result in Ca^{2+} extrusion via the PMCA that activates CaRs on the same cell (or adjacent cell) through local increases in $[Ca^{2+}]_{out}$. This would reinforce the initial stimulus, promoting the initiation of a second spike in intracellular Ca^{2+}.

In order to test this hypothesis, cells were seeded onto special glass coverslips, as described previously (Hofer et al., 2000), and allowed to grow underneath small lacunae created between fused polypropylene microfibers and the glass of the coverslip (see Materials and Methods for details). In this way cells were maintained in a restricted extracellular volume shielded from the bulk solution, as an approximation of the limited extracellular volumes found in intact tissues.

We first tested the effects of extracellular Ca^{2+} buffers on CaR-mediated Ca^{2+} signaling. HEK CaR-expressing cells were stimulated with 1 mM spermine in nominally extracellular free Ca^{2+} to which BAPTA free acid (used at concentrations ranging from 10 to 60 μM) was added to the bath to reduce [Ca^{2+}] to very low levels (from 175 nM to 17 nM, respectively). Under these conditions spermine was still able to induce repetitive intracellular Ca^{2+} spikes (Fig. 1A). The intracellular [Ca^{2+}] at the peak of the initial spike was estimated to be 410.7±21.5 nM, starting from a resting value that averaged 73.8±6.6 nM (*n*=5 calibrated experiments; data from 93 cells). Oscillations were sustained for 5-10 minutes until they gradually dampened out (typical of *n*=19 independent experiments; 347 cells), presumably due to the depletion of intracellular stores that could not be refilled by calcium entry mechanisms (i.e. store-operated channels, or SOCs). This demonstrates that oscillations mediated by CaR resulted from the release of Ca^{2+} from intracellular stores triggered by CaR and did not depend, at least over the short-term, on entry of external Ca^{2+}. However, as seen in Fig. 1B, increasing BAPTA to 1 mM (thereby greatly increasing the external buffering capacity for Ca^{2+}, while still maintaining free Ca^{2+} at a little less than 2 nM) reversibly blocked or attenuated intracellular spiking (*n*=7; 65 cells). In other experiments (not shown), the presence of 1 mM BAPTA did not significantly alter the speed or magnitude of the initial spike of Ca^{2+} following spermine addition as compared to the spike in the same cells in the presence of 50 μM BAPTA (*n*=5 experiments; 85 cells). Thus it does not appear that BAPTA interferes with the binding of the agonist to CaR. To ensure that the difference in the oscillatory response was not due to the fact that [Ca^{2+}] was slightly lower in the solution containing 1 mM BAPTA as compared to the solutions containing 10-60 μM BAPTA, experiments were also performed in solutions where free [Ca^{2+}] was matched but the total [BAPTA] was varied (see Materials and Methods for details). Fig. 1C compares a control stimulation to spermine in nominally Ca^{2+}-free Ringer's containing 10 μM BAPTA (measured free [Ca^{2+}] approximately 150 nM) to the response in a solution containing 1 mM BAPTA and 0.49 mM $CaCl_2$ (measured free [Ca^{2+}] approx. 200 nM). Oscillations were consistently attenuated in the highly buffered solution, even though the free [Ca^{2+}] was actually slightly elevated in that solution (typical of *n*=4 experiments; 93 cells).

A variation of this experiment in the presence of higher external [Ca^{2+}] is shown in Fig. 1D,E, in which 20 mM citrate was used as the Ca^{2+} buffer, allowing an external [Ca^{2+}] that more closely matches physiological concentrations (i.e. around 1 mM Ca^{2+}). Ca^{2+} signals stimulated by spermine were compared in the same cells under control conditions (1 mM added Ca^{2+} in the bath) with those elicited in the presence of the Ca^{2+} buffer (with the free Ca^{2+} matched to the control solution as described in Materials and Methods). As seen in Fig. 1D, the presence of the buffer dramatically reduced the frequency of the oscillations (*n*=2, 56 cells). As shown in Fig. 1E, this effect was reversible (*n*=4, 85 cells). Cells were initially stimulated with spermine in a control Ringer's that contained 0.65 mM added Ca^{2+}, and the solution switched between buffered and unbuffered during oscillatory spiking (the lower [Ca^{2+}] was used in these later experiments because it more closely matches the K_d of citrate for Ca^{2+}, but this did not significantly affect the Ca^{2+} buffering power of the solution). There was a marked effect of the buffer on both the amplitude and frequency of the oscillations. In contrast, stimulation of HEK CaR cells with carbachol and ATP (to activate endogenous muscarinic and purinergic receptors, respectively) in the presence of the citrate buffer yielded a response that was similar to control (Fig. 1F), demonstrating that the Ca^{2+} buffer itself did not have deleterious effects on cellular Ca^{2+} signaling machinery. Moreover, some cells were observed to oscillate (on top of an elevated plateau), showing that Ca^{2+} spiking elicited by carbachol/ATP was insensitive to the citrate buffer (typical of *n*=4 experiments, 70 cells). The buffering power of the citrate solution used in the experiment shown in Fig. 1D is shown in Fig. 1G.

In a second type of study, we explored the role of the PMCA in the maintenance of intracellular oscillations. As shown in Fig. 2A, 100 nM $HgCl_2$, a potent but highly nonspecific PMCA antagonist that does not interact with fura-2, and is not a ligand of the CaR, was added acutely to oscillating cells. $HgCl_2$ blocked oscillations mediated by spermine in Ca^{2+} free solutions (*n*=3 experiments, 42 cells), and also blocked oscillations elicited by NPS-R-467 (5 μM) in the presence of

1 mM Ca^{2+} (not shown; *n*=2 experiments, 28 cells). As shown in Fig. 2B, the compound did not affect the initial release of Ca^{2+} from stores (typical of *n*=4 experiments, 25 cells). In contrast to the actions of $HgCl_2$ on CaR-mediated oscillations, the agent did not appreciably block repetitive spiking induced by carbachol/ATP in HEK CaR cells, as shown in Fig. 2C. This

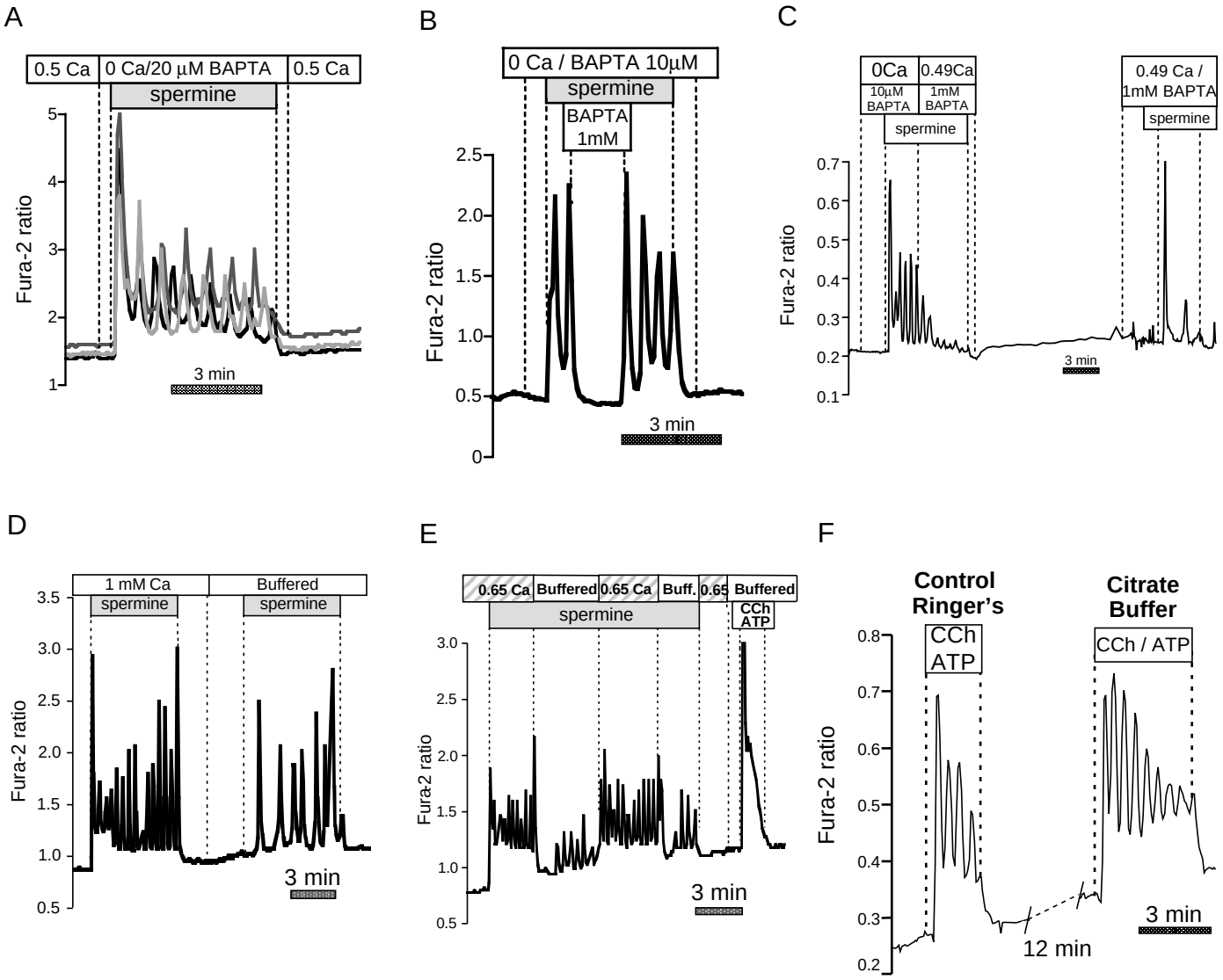

Fig. 1. (A) HEK CaR cells can oscillate for up to 10 minutes in the absence of external Ca^{2+} (10-20 μM BAPTA free acid in bath) following stimulation with the CaR agonist spermine (1 mM). Traces from several representative cells are shown. Time bar indicates three minutes. (B) Increasing the BAPTA concentration from 10 μM to 1 mM reversibly interrupts cytosolic Ca^{2+} signals (oscillations) stimulated by 1 mM spermine. (C) Comparison of oscillations induced by 1 mM spermine in nominally Ca^{2+}-free Ringer's containing 10 μM BAPTA vs a solution containing 1 mM BAPTA + 0.49 mM $CaCl_2$. The free Ca^{2+} in the 10 μM BAPTA solution (approx. 150 nM) was directly measured to be slightly lower than that in the 1 mM BAPTA/0.49 mM $CaCl_2$ solution (approx. 200 nM; see Materials and Methods for details). (D) Oscillations elicited by spermine (1 mM) in the presence of 1 mM Ca^{2+} were attenuated in a buffer containing 20 mM citrate (added to buffer extracellular Ca^{2+} changes) plus approx. 10 mM $CaCl_2$. Free Ca^{2+} was the same in control and buffered solutions, and was titrated using a calcium-selective electrode as described in Materials and Methods. (E) Frequency and amplitude of oscillatory signals elicited by spermine in the presence of 0.65 mM added Ca^{2+} were reversibly altered by citrate-buffered solution. As in panel D, the solution containing 20 mM citrate plus approx. 8 mM $CaCl_2$, contained the same free [Ca^{2+}] as the control solution, matched using a Ca^{2+} electrode. (F) Oscillations in HEK CaR cells stimulated by 100 μM carbachol (CCh) and 100 μM ATP were not affected by the same citrate buffer used in Fig. 1E. (G) Plot of Ca^{2+} buffering capacity of citrate solution used in Fig. 1D. Top trace, unbuffered (control) solution showing free [Ca^{2+}] change as measured with a Ca^{2+}-electrode with the addition of 600 μM increments of added $CaCl_2$; bottom trace, parallel measurement with citrate-containing solution. The buffering characteristics of the solutions used in Fig. 1E containing 0.65 mM Ca^{2+} and 20 mM citrate plus $CaCl_2$ were similar (not shown).

record also illustrates that $HgCl_2$ is an effective inhibitor of the PMCA. The initial peak following carbachol/ATP stimulation was significantly (P<0.0001) greater in the presence of $HgCl_2$ (120% of control in the same cell; peak ratio 0.779±0.017 for control vs. 0.9435±0.0331 with $HgCl_2$; n=3 experiments; data from 35 cells), and the return to baseline following Ca^{2+} removal in the continued presence of agonist (largely a measure of PMCA-mediated extrusion) was likewise significantly retarded, by more than two-fold (relative slope of 0.0020±0.0002 for control vs. 0.0009±0.001 in the presence of $HgCl_2$; P<0.0001). We also tested the effect of $HgCl_2$ on Ca^{2+} spiking induced by low concentrations of carbachol (10 μM)

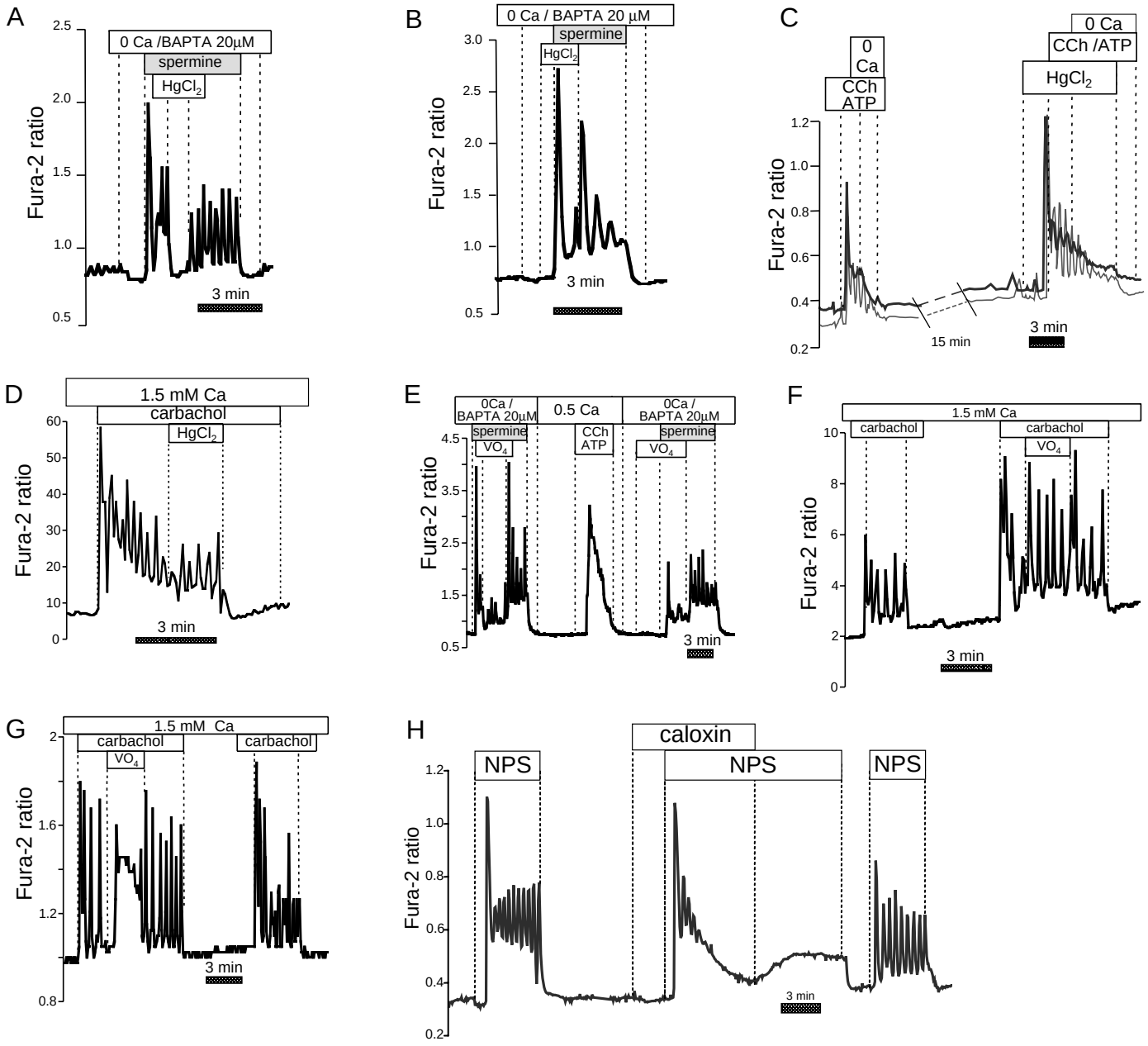

Fig. 2. (A) HEK CaR cells were stimulated with 1 mM spermine in the absence of extracellular Ca^{2+}. Addition of 100 nM $HgCl_2$ produced a rapid and reversible block of Ca^{2+} spiking. (B) Pretreatment with 100 nM $HgCl_2$ did not stimulate HEK CaR cells by itself or prevent the release of Ca^{2+} from internal stores induced by 1 mM spermine. (C) Oscillations resulting from stimulation with carbachol (CCh; 100 μM) and ATP (100 μM) were not blocked by 100 nM $HgCl_2$ in HEK CaR cells. These records also illustrate that Hg^{2+} is an effective blocker of the PMCA in HEK CaR cells, since the time to return to baseline following Ca^{2+} removal (in the presence of agonists) was significantly attenuated by $HgCl_2$. (D) Ca^{2+} spiking was not abolished by 100 nM $HgCl_2$ in HEK WT cells stimulated with 10 μM carbachol. (E) Spermine-induced oscillations were attenuated by 1 mM orthovanadate in HEK CaR cells. (F) Vanadate produced one of two effects on carbachol-stimulated oscillations in HEK WT cells. Shown here are cells in which 1 mM vanadate had little or no action on oscillations (42% of cells). (G) Vanadate (1 mM) sometimes produced an elevated plateau of Ca^{2+} when added acutely to carbachol-stimulated HEK WT cells (58% of cells). (H) 2 mM Caloxin 2A1, a peptide inhibitor of the PMCA, blocked oscillations elicited by NPS R-467 (5 μM).

in HEK293 WT cells. The phenomenon of baseline spiking induced by low doses carbachol in HEK WT cells has been well studied, and seems to be highly dependent on influx of Ca^{2+} through entry pathways that continue to be characterized (Luo et al., 2001; Shuttleworth, 1996). Thus 1.5 mM Ca^{2+} was present in the external medium for these control experiments on WT cells, which showed that $HgCl_2$ caused a slight slowing of carbachol-induced oscillations (Fig. 2D; *n*=8, 98 cells). This effect may be the consequence of inhibition of Ca^{2+} entry by $HgCl_2$, which is known to block some Ca^{2+} channels. This effect was very different, however, from the fast inhibition observed in the CaR-expressing cells shown in Fig. 2A.

The actions of a different PMCA inhibitor, sodium orthovanadate (Qi et al., 2000), are shown in Fig. 2E. Since this compound acts on a cytoplasmic site of the pump, relatively high concentrations (2 mM) were used to insure penetration into cells. Ca^{2+} oscillations in HEK CaR cells elicited by 1 mM spermine in Ca^{2+}-free solutions were significantly blocked by addition of VO_4^{3-} to the bath (*n*=4, 46 cells). In contrast, in control experiments (Fig. 2F), Ca^{2+} spiking induced in non-transfected HEK293 WT cells by 10 μM carbachol was completely unaffected in 42% of the cells by addition of vanadate to the external solutions. In the remaining 58% of cells, however, an elevated plateau phase was observed as illustrated in Fig. 2G (*n*=6, 153 cells total). This latter effect might be partially explained by inhibitory actions of vanadate on the SERCA (Sarco-Endoplasmic Reticulum Calcium ATPase), which would prevent Ca^{2+} clearance from the cytoplasm into the ER, and lead to greater entry of Ca^{2+} though SOCs by preventing store refilling. Overall it appears that PMCA inhibition does not have a dramatic effect on oscillations in WT cells. This conclusion is consistent with previous reports of Morgan and Jacob (Morgan and Jacob, 1998) who found that inhibiting the PMCA with La^{3+} (which could not be used in the present study because it is an agonist of CaR) had little effect on Ca^{2+} oscillations in endothelial cells. Moreover, Green et al., also showed that another PMCA inhibitor, carboxyeosin (which also could not be used here due to its intense fluorescence) did not block Ca^{2+} oscillations in rat hepatocytes as measured by the luminescent photoprotein aequorin (Green et al., 1997).

The PMCA inhibitors $HgCl_2$ and VO_4^{3-} are not ideal. $HgCl_2$ unfortunately has many well-recognized cytotoxic effects, including induction of free radical formation, lipid peroxidation, and other actions resulting from its ability to form strong bonds with –SH groups of proteins. Likewise, VO_4^{3-} inhibits all P-type ATPases, including the SERCAs. Breitwieser and Gama (Breitwieser and Gama, 2001) demonstrated that exposure of HEK293 CaR-expressing cells to thapsigargin (1 μM), a specific blocker of SERCAs, completely eliminated Ca^{2+} oscillations induced by increasing $[Ca^{2+}]$ in extracellular bath from 2 to 3.5 mM (although the presence of elevated Ca^{2+} in the bath might obscure the effects on oscillations due to the increased entry of Ca^{2+}). Nevertheless the experiments with VO_4^{3-} must be interpreted with caution, due to the inhibitory effects of this agent on SERCAs. We therefore tested a more specific PMCA antagonist, Caloxin 2A1, a recently described peptide inhibitor comprised of 12-amino acids (Chaudhary et al., 2001).

Caloxin 2A1 is active at an extracellular site, so the peptide can simply be added exogenously to inhibit the PMCA. Caloxin has a relatively low-affinity for the pump (K_i=0.4 mM; complete inhibition at 2 mM) (Chaudhary et al., 2001). Therefore cells were pretreated for one minute with the peptide prior to stimulating the cells. As seen in Fig. 2H, under control conditions, activation of CaR with 5 μM NPS R-467 in the presence of 1 mM Ca^{2+} produced an initial transient in Ca^{2+} followed by repetitive Ca^{2+} spikes superimposed on a sustained plateau. The spiking did not diminish during agonist exposure. In contrast, following Caloxin 2A1 pretreatment, NPS R-467

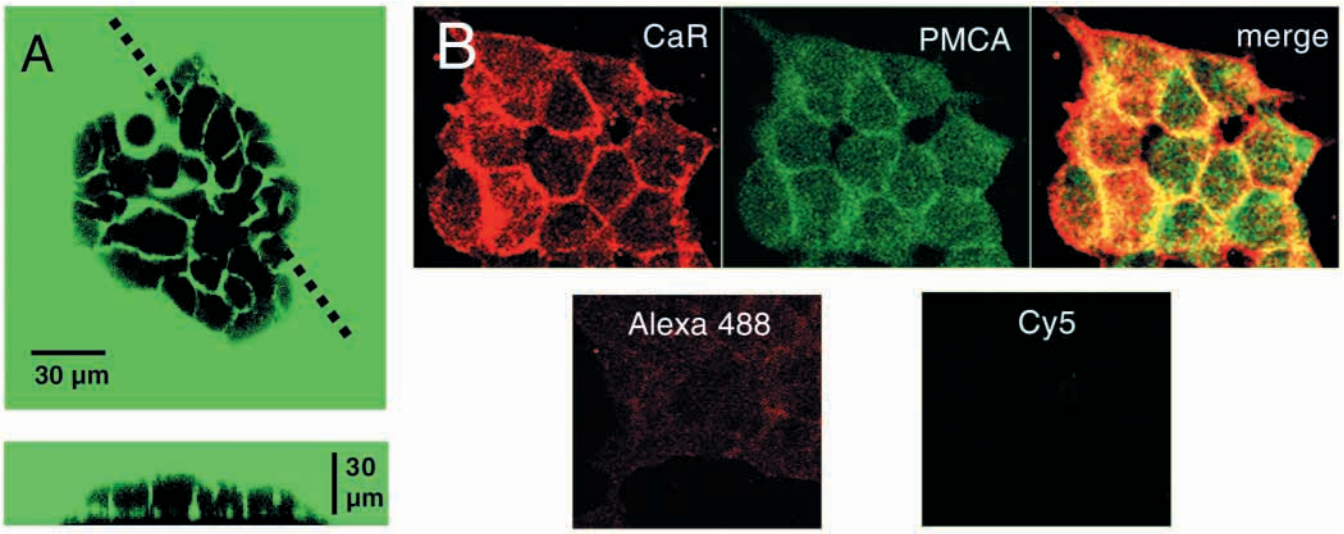

Fig. 3. (A) Top, confocal *xy*-section through center of a cluster of living HEK CaR cells incubated with an extracellular fluorescent marker (fluorescein-dextran; 10,000 MW) illustrating limited aqueous spaces between cells. Bottom, 'side-view' profile (*yz*) reconstructed from stack of confocal z-sections (each 1 μm thick) through the region indicated by the line shown in the top image (line width=3 pixels or ~1.5 μm). (B) Co-immunostaining of CaR and PMCA in HEK CaR cell clusters. Top left, Alexa 488 staining of CaR (red pseudocolor); middle, CY5 labeling of PMCA (green pseudocolor); top right (merged image), yellow color depicts areas of overlap. Bottom panels show control staining for Alexa 488 + CaR peptide block (see Materials and Methods for details) and Cy5 secondary antibody alone.

elicited a similar initial spike, and then a few feeble oscillations that gradually decayed towards the baseline. Spiking was sometimes restored upon removal of the peptide inhibitor (not shown), but more frequently there was a modest increase in the plateau without oscillations. Oscillatory behavior was recovered following a third (control) stimulation (n=5; 41 cells). Acute addition of 2 mM Caloxin 2A1 during oscillations stimulated by 1 mM spermine in Ca^{2+}-free Ringer's (20 μM BAPTA) also attenuated spiking (n=3 experiments, 51 cells; not shown).

Thus far our data suggest a role for PMCA-mediated Ca^{2+} extrusion in providing an additional extracellular stimulus to CaR that results in intracellular Ca^{2+} spiking. As shown in Fig. 3A, the physical architecture of these cells may also contribute to this phenomenon. Optical sectioning through HEK-CaR cell clusters bathed with membrane-impermeant fluorescein-dextran (10,000 MW) showed that the junctions between cells consisted of very limited aqueous spaces. These convoluted clefts extended to the bottom of the cell clusters, as illustrated by the reconstructed side-view image, where immunostaining for CaR and PMCA showed that these two membrane proteins were also present. As seen in the confocal section in Fig. 3B, there was substantial overlap between PMCA and CaR (shown by yellow color in the merged image) in these junctional areas. The PMCA, in particular, was frequently observed to be absent from the free edges of the cell clusters.

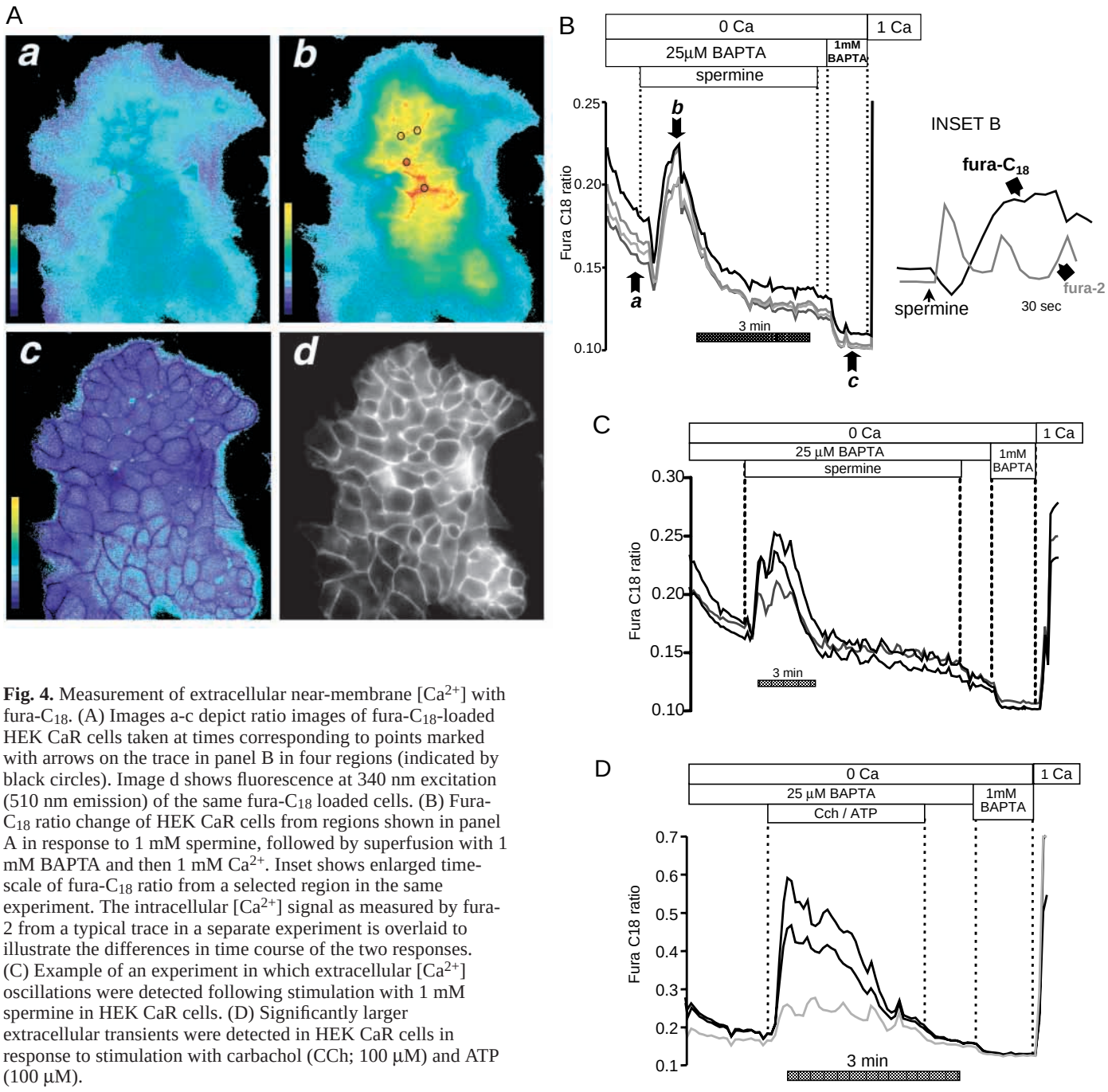

Fig. 4. Measurement of extracellular near-membrane $[Ca^{2+}]$ with fura-C_{18}. (A) Images a-c depict ratio images of fura-C_{18}-loaded HEK CaR cells taken at times corresponding to points marked with arrows on the trace in panel B in four regions (indicated by black circles). Image d shows fluorescence at 340 nm excitation (510 nm emission) of the same fura-C_{18} loaded cells. (B) Fura-C_{18} ratio change of HEK CaR cells from regions shown in panel A in response to 1 mM spermine, followed by superfusion with 1 mM BAPTA and then 1 mM Ca^{2+}. Inset shows enlarged time-scale of fura-C_{18} ratio from a selected region in the same experiment. The intracellular $[Ca^{2+}]$ signal as measured by fura-2 from a typical trace in a separate experiment is overlaid to illustrate the differences in time course of the two responses. (C) Example of an experiment in which extracellular $[Ca^{2+}]$ oscillations were detected following stimulation with 1 mM spermine in HEK CaR cells. (D) Significantly larger extracellular transients were detected in HEK CaR cells in response to stimulation with carbachol (CCh; 100 μM) and ATP (100 μM).

We attempted to evaluate whether co-localization of PMCA and CaR in membrane microdomains such as caveolae might be important for generating Ca^{2+} oscillations. HEK CaR cells were therefore pre-incubated with β-cyclodextrin (see Materials and Methods) to in order to disrupt caveolae. While this treatment inhibited spermine- and NPS-R-467-induced oscillations in most cells, these experiments were inconclusive because β-cyclodextrin also abolished the initial spike of Ca^{2+} release in 52% of the cells following addition of the CaR agonists (n=4 experiments; 134 cells; data not shown). In the same cells the response to carbachol/ATP was also absent.

Additional support for our model, however, derives from the time course for extracellular [Ca^{2+}] changes following agonist activation, as measured directly using the near-membrane Ca^{2+} probe fura-C_{18} (Etter et al., 1994). When incubated with intact cells, near-membrane indicators become anchored to the external leaflet of the plasma membrane via a lipid tail, and have been used previously to measure Ca^{2+} extrusion from cells (Blatter and Niggli, 1998; Nitschke et al., 1997). The fluorescence of dye-loaded cells was quenched completely by addition of 5 mM $NiCl_2$ (which is cell impermeant), confirming the extracellular location of the fluorophore (see Materials and Methods for details). Because fura-C_{18} is a high affinity Ca^{2+} probe (K_d=150 nM) (Etter et al., 1994), we were obliged to conduct our experiments in nominally Ca^{2+}-free solutions containing 25 μM BAPTA free acid.

Stimulation of HEK CaR cells with 1 mM spermine resulted in a relatively slow peak in extracellular [Ca^{2+}] that originated from the junctional regions in the center of cell clusters (Fig. 4A). Several different patterns of extracellular [Ca^{2+}] change were apparent. In 6 out of 19 experiments, there was a small initial undershoot in the Fura-C_{18} ratio (illustrated in Fig. 4B), followed by a larger elevation that peaked 34.8±1.3 seconds after stimulation (data from 6 experiments; junctional regions between 64 cells). This was significantly slower than the time to attain the peak in cytoplasmic Ca^{2+} following spermine stimulation, as measured by fura-2 under the same experimental conditions (10.0±0.4 seconds; data from 73 cells). The time course of a typical cytoplasmic spike is overlaid on the fura-C_{18} trace at an expanded scale in the inset of Fig. 4B to illustrate this point. In five (out of 19) other experiments the initial undershoot was absent leaving just the slow transient elevation. Treatment of cells with 1 mM VO_4^{3-} (to block the PMCA) prevented the spermine-induced peak, unmasking only the undershoot (typical of n=6 experiments; not shown). In some experiments (8 out of 19) oscillatory fluctuations in extracellular [Ca^{2+}] were detected following spermine addition, as shown in Fig. 4C. Significantly larger elevations in extracellular [Ca^{2+}] (which were also sometimes oscillatory) were observed in response to other Ca^{2+}-mobilizing agonists, carbachol and/or ATP (Fig. 4D). ATP and other agonists have been reported previously to activate the PMCA in HEK293 cells (Qi et al., 2000) and other cell types (Usachev et al., 2002).

Discussion

In the present study we provide evidence for a novel mechanism that may serve to reinforce Ca^{2+} signals (manifested as Ca^{2+} oscillations) in cells that express the extracellular calcium-sensing receptor (CaR). Oscillations were sensitive to PMCA inhibitors and extracellular Ca^{2+} buffers. Our direct measurements of extracellular near-membrane [Ca^{2+}] indicated that local external [Ca^{2+}] peaked about 35 seconds after stimulation with spermine and slowly dissipated. It appears likely that extruded Ca^{2+} contributes to the basal activation of CaR following the initial intracellular Ca^{2+} transient, and this is then translated into Ca^{2+} oscillations inside the cell by other 'classical' mechanisms involving, for example, the sensitization of $InsP_3$ receptors by Ca^{2+}, or through cycles of PKC-mediated phosphorylation. In some experiments oscillatory fluctuations in extracellular [Ca^{2+}] were detected. This may potentially contribute directly to repetitive activation of the receptor. Collectively, these data are consistent with a model in which the cycling of Ca^{2+} across the plasma membrane by the PMCA increases [Ca^{2+}] in local extracellular microdomains and potentiates the actions of CaR agonists (Fig. 5).

Our immunolocalization data showed that in multi-cellular clusters of HEK CaR cells, the PMCA was largely restricted to junctures between cells (Fig. 3B), where it co-localized with CaR. Confocal sectioning of living cell clusters incubated with membrane-impermeant fluorescent dextrans revealed that an aqueous cleft occupies these junctions between cells, as the fluorescent marker was able to penetrate into these spaces (Fig. 3A). Thus it would appear that Ca^{2+} is expelled from the cell by the PMCA into highly restricted extracellular domains, as confirmed by our direct measurements of extracellular [Ca^{2+}]. This may help to explain why single cells oscillated less vigorously than those growing in clusters. In parathyroid cells (which express CaR very abundantly) the receptor is known to localize to caveolae (Kifor et al., 1998), small invaginations in the plasma membrane that have been considered to be cellular 'signaling centers'. The PMCA has also been localized to caveolae (Fujimoto, 1993; Ogi et al., 2000) in other cell types. Unfortunately, our attempts to assess the role of caveolae in Ca^{2+} oscillations using β-cyclodextrin were inconclusive because the compound frequently altered the initial spike of Ca^{2+} in response to agonists. Thus it remains to be determined

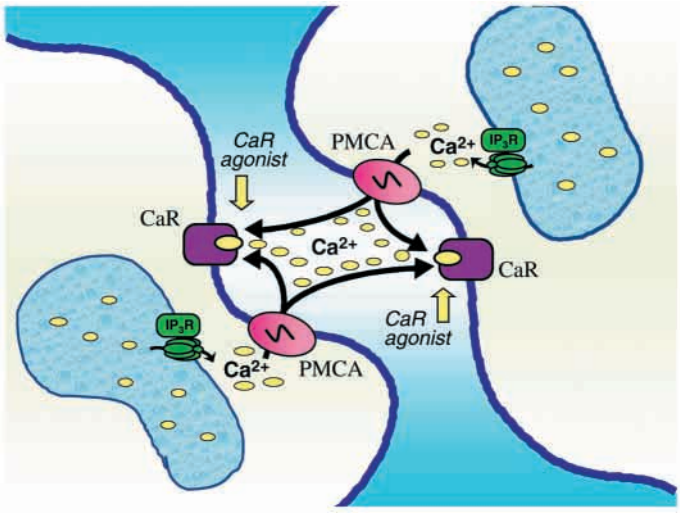

Fig. 5. Model showing proposed mechanism by which the PMCA might provide positive feedback on CaR of the same cell or neighboring cells, reinforcing signaling via CaR.

whether CaR and PMCA may reside together in the same specialized membrane domain, and how this may influence the signaling properties of the cell.

It is noteworthy that parathyroid cells have been observed to oscillate 'spontaneously' in the presence of elevated extracellular Ca^{2+} (Miki et al., 1995). 'Spontaneous' Ca^{2+} oscillations have also been observed in number of other diverse cell types, including human osteoblast-like cells (hOB cells) (Tsai et al., 1999), the pancreatic glucagon-secreting cell line INR1 G9 (Bode et al., 1994), mouse mammary epithelial cells, both in primary culture and in an established cell line MMT060562 (Furuya et al., 1993), isolated secretory ciliary epithelium (Giovanelli et al., 1996), and primary rat mammotropes (Shorte et al., 2000). CaR is a widely expressed receptor, but is not known whether it may play any role in activating Ca^{2+} spiking in these latter cell types. Moreover, additional studies will be needed to determine whether the mechanism described here in the HEK CaR model will be functional in cell types where CaR is expressed endogenously.

Of particular interest, however, are 'spontaneous' Ca^{2+} oscillations in the developing nervous system that have been shown to be mediated by metabotropic glutamate receptors (Flint et al., 1999). Certain members of the group I mGluR family, specifically subtypes mGluR1 and mGluR5, are linked to PLC/$InsP_3$/Ca^{2+} signaling pathways (Hermans and Challiss, 2001). CaR in fact bears significant molecular homology with mGluRs (Brown et al., 1993). Moreover, mGluR1a and mGluR5a have been suggested to have Ca^{2+}-sensing capacity (EC_{50} for Ca^{2+} approx. 1 mM), and can apparently utilize Ca^{2+} as a co-agonist (Kubo et al., 1998; Nash et al., 2001a). Considering the putative Ca^{2+}-sensing properties of these receptors (particularly mGluR5a), it is conceivable that cycling of Ca^{2+} across the plasma membrane may also contribute to stimulation of the mGluR in the presence of an agonist, subtly amplifying the stimulus for oscillatory signaling.

The role of PKC in mGluR-mediated oscillations has been investigated extensively. Meyer and colleagues (Codazzi et al., 2001) recently re-examined this complex phenomenon in astrocytes (which express mGluR5 endogenously) using total internal reflection fluorescence imaging of GFP-tagged PKCδ and GFP-tagged C1 domains of PKCδ. The latter construct binds DAG, and can be used as a reporter of [DAG] as well as to buffer its concentration. DAG was found to oscillate along with Ca^{2+} following glutamate stimulation, and over-expression of the DAG binding domain altered the ability of the cell to return to baseline during the downstroke of the Ca^{2+} signal. PKC may therefore be important for the termination of the Ca^{2+} spike. However the various factors contributing to the initiation of the oscillatory spike have not been fully elucidated.

While it is tempting to speculate that Ca^{2+} cycling across the plasma membrane is the primary driving force behind the oscillatory signal, our data and those of others suggest that additional mechanism (s) apart from PMCA-mediated cycling of Ca^{2+} likely contribute to sustaining oscillations in HEK CaR cells. In spite of treatments that would be expected to inhibit the PMCA or buffer external Ca^{2+} completely, repetitive Ca^{2+} spiking was not always fully abolished (e.g. Fig. 2E,H). Moreover, our direct measurements of extracellular [Ca^{2+}] using fura-C_{18} showed that the extracellular [Ca^{2+}] increase gradually dissipated at a time point well before intracellular oscillations were expected to cease under Ca^{2+}-free conditions. Mutational analyses of CaR have shown that consensus phosphorylation sites for PKC are important for shaping the intracellular Ca^{2+} signal arising from CaR stimulation (Bai et al., 1998; Chang et al., 1999). In a study using CaR expressing oocytes Chang et al., showed that $^{45}Ca^{2+}$ efflux (used by the authors as a measure of CaR activation) was susceptible to PMA treatment (Chang et al., 1999). Meanwhile, interpretation of these studies is complicated by the fact that PKC may also modulate some isoforms of the PMCA (Enyedi et al., 1997; Penniston et al., 1997; Qu et al., 1992; Zylinska et al., 1998). A role for PKC in CaR-mediated Ca^{2+} oscillations has been suggested by several studies. For example, Breitwieser and Gama (Breitwieser and Gama, 2001) found that PKC inhibition (e.g. with staurosporine) caused cells to be more likely to oscillate (although the inter-spike frequency was similar to control). In our own unpublished studies, stimulation of PKC using a brief application of PMA (4β-phorbol 12 myristate 13-acetate; 100 nM) abolished CaR-dependent Ca^{2+} oscillations in HEK CaR cells (Y. Jiang, A.M.H., B. W. Lau and M. Bai, unpublished). Thus, for CaR-expressing cells, it is possible that activated PKC is somehow involved in terminating individual Ca^{2+} spikes, as was suggested for mGluR-expressing cells.

CaR desensitizes relatively slowly (Breitwieser and Gama, 2001; Gama and Breitwieser, 1998), yet physiological conditions may exist in which the receptor is chronically exposed to elevated levels of CaR activators. Ca^{2+} spiking is a mode of signaling that allows continuous encoding of information while avoiding the potentially deleterious effects of persistent elevations in cytoplasmic [Ca^{2+}], as well as the harmful effects of chronic depletion of Ca^{2+} in the endoplasmic reticulum. We report here that appropriately timed cycling of Ca^{2+} across the plasma membrane by the PMCA may assist in the initiation of the oscillatory spike by amplifying the actions of other CaR agonists (including extracellular Ca^{2+}). We speculate that in CaR expressing cells (and possibly also in cells harboring other Ca^{2+} sensors such as mGluRs) several mechanisms may coexist, converging to reinforce oscillatory behavior. These seemingly redundant mechanisms may be important to ensure that extracellular signals are translated into Ca^{2+} oscillations, even in the face of prolonged exposure to maximal agonist concentrations.

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