Differential Regulation of the Apical Plasma Membrane Ca\(^{2+}\)-ATPase by Protein Kinase A in Parotid Acinar Cells*

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Cross-talk between intracellular calcium ([Ca\(^{2+}\)]\(_{\text{i}}\)) signaling and cAMP defines the specificity of stimulus-response coupling in a variety of cells. Previous studies showed that protein kinase A (PKA) potentiates and phosphorylates the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) in a Ca\(^{2+}\)-dependent manner in parotid acinar cells (Bruce, J. I. E., Yule, D. I., and Shuttleworth, T. J. (2002) J. Biol. Chem. 277, 48172–48181). The aim of this study was to further investigate the spatial regulation of [Ca\(^{2+}\)]\(_{\text{i}}\), clearance in parotid acinar cells. Par-C10 cells were used to functionally isolate the apical and basolateral PMCA activity by applying La\(^{3+}\) to the opposite side to inhibit the PMCA. Activation of PKA (using forskolin) differentially potentiated apical [Ca\(^{2+}\)]\(_{\text{i}}\), clearance in mouse parotid acinar cells and apical PMCA activity in Par-C10 cells. Immunofluorescence of parotid tissue slices revealed that PMCA1 was distributed throughout the plasma membrane, PMCA2 was localized to the basolateral membrane, and PMCA4 was localized to the apical membrane of parotid acinar cells. However, in situ phosphorylation assays demonstrated that PMCA1 was the only isoform phosphorylated by PKA following stimulation. Similarly, immunofluorescence of acutely isolated parotid acinar cells showed that the regulatory subunit of PKA (RI\(\beta\)) translocated to the apical region following stimulation. These data suggest that PKA-mediated phosphorylation of PMCA1 differentially regulates [Ca\(^{2+}\)]\(_{\text{i}}\) clearance in the apical region of parotid acinar cells because of a dynamic translocation of PKA. Such tight spatial regulation of Ca\(^{2+}\) efflux is likely important for the fine-tuning of Ca\(^{2+}\)-dependent effectors close to the apical membrane important for the regulation of fluid secretion and exocytosis.

Different spatial and temporal “shapes” of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{i}}\)\(^{2}\) signals encode a vast array of cellular information and underlie stimulus-response coupling in almost every cell type (1, 2). This “spatio-temporal shaping” of [Ca\(^{2+}\)]\(_{\text{i}}\) signals can be achieved by the concomitant activation of multiple signaling pathways. For example, elevation of cAMP, and the subsequent activation of protein kinase A (PKA), directly modulates the activity or spatial organization of key Ca\(^{2+}\) transport proteins in many nonexcitable cells (3). Parotid acinar cells represent an excellent model system to study such signaling cross-talk because effective fluid secretion in these cells is critically dependent on the exquisite spatio-temporal control of [Ca\(^{2+}\)]\(_{\text{i}}\) signals by elevation of cAMP. Such signaling cross-talk is believed to be important for the precise control of apically located Ca\(^{2+}\)-dependent Cl\(^{-}\) channels and basolaterally located Ca\(^{2+}\)-dependent K\(^{+}\) channels that maintain maximum ion and thus water movement (4, 5). Previous studies in acutely isolated parotid acinar cells have demonstrated that the key molecular mechanisms for this signaling cross-talk are the PKA-mediated modulation of inositol 1,4,5-trisphosphate receptors (IP\(_{3}\)R), which control Ca\(^{2+}\) release, and plasma membrane Ca\(^{2+}\)-ATPase (PMCA), which control [Ca\(^{2+}\)]\(_{\text{i}}\), clearance (6, 7). Specifically, PKA potentiates and phosphorylates the PMCA but only in the presence of [Ca\(^{2+}\)]\(_{\text{i}}\)-raising agents (7). Taken together these may represent an important mechanism by which cAMP tightly controls the spatial and temporal properties of Ca\(^{2+}\) signaling and thus the potentiation of fluid secretion by cAMP-raising agonists in the parotid.

The aim of this study was to further explore the molecular mechanisms for the spatial control of [Ca\(^{2+}\)]\(_{\text{i}}\), clearance by cAMP. This was achieved by imaging acutely isolated mouse parotid acinar cells and Par-C10 cells (immortalized rat parotid acinar cell line (8)). These cells were used as a convenient model for functionally isolating apical from basolateral PMCA activity by separately perfusing the corresponding side with La\(^{3+}\). The study revealed that the apical PMCA was differentially potentiated by activation of PKA despite being expressed in all regions of the membrane. Such tight spatial regulation of Ca\(^{2+}\) efflux may represent an important mechanism for the fine-tuning of Ca\(^{2+}\)-dependent effectors at the apical membrane important for the regulation of fluid secretion and exocytosis.

**EXPERIMENTAL PROCEDURES**

**Isolation of Mouse Parotid Acinar Cells**—Parotid acinar cells were isolated into small clusters by collagenase digestion as described previously (6). Following isolation, cells were resuspended in collagenase digestion—but not NCX, Na\(^{+}\)-Ca\(^{2+}\) exchange; NMDG, N-methyl-D-glucamine; AKAP, protein kinase A anchoring protein.
pared in a HEPES-buffered physiological saline solution (HEPES-PSS) containing (in mM) 5.5 glucose, 137 NaCl, 0.56 MgCl₂, 4.7 KCl, 1 Na₂HPO₄, 10 HEPES (pH 7.4), 1.2 CaCl₂. Cells were resuspended in HEPES-PSS containing 1% bovine serum albumin (BSA, Sigma) and kept at 4 °C until ready for use.

Par-C10 Cell Culture—Par-C10 cells were generously provided by Dr. David Quissel (School of Dentistry, University of Colorado Health Sciences Center, Denver). Cells were grown either on glass coverslips (25 mm, VWR Scientific) or on permeable clear polyester Transwell supports (0.33 cm², pore size 0.4 μm; Corning Costar). The cultures were grown to confluence at 37 °C in a humidified 95% air, 5% CO₂ atmosphere in DMEM/F-12 (1:1) (Sigma) containing 10% filtered fetal bovine serum (Invitrogen) and the following supplements: 1 μM retinoic acid, 2 mM triiodothyronine, 0.4 μg/ml hydrocortisone (all from Sigma), 500 units/ml penicillin, and 0.5 mg/ml streptomycin (Invitrogen). Cells grown on glass coverslips were used typically 4–5 days after plating, whereas cells grown on the Transwell inserts were left to differentiate for 10–15 days prior to experimentation. This was found empirically to be the minimum period required for the cells to form tight junctions as assessed by measuring the transepithelial electrical resistance (TEER), using an EVOM epithelial voltohmmeter with chopstick probes (WPI, Stevenage, Herts, UK).

**Digital Imaging Fura-2 Fluorescence in Mouse Parotid Acinar Cells**—Cells were loaded with 2 μM fura-2-acetoxymethyl ester (TEF Labs) in HEPES-PSS for 30 min at room temperature (see Ref. 7). Dye-loaded cells were allowed to adhere to a glass coverslip that formed the base of a gravity-fed perfusion chamber, continually perfused with HEPES-PSS, and automatic valves were used for rapid switching of solutions (Harvard Apparatus Ltd., Kent, UK). Fluorescence imaging experiments were performed using an inverted epifluorescence Nikon TE2000 microscope with ×40 oil immersion objective (NA, 1.3) attached to a CoolSNAP HQ interline progressive-scan charged coupled device camera (Roper Scientific Photometrics, Tucson, AZ) and monochromator illumination system (Cairn Research, Kent, UK). Image acquisition and analysis were all controlled by MetaFluor/MetaMorph imaging software (Molecular Devices, Downingtown, PA). Background-subtracted 340 and 380 nm fluorescence images were captured with no binning at a rate of 1 Hz, and 340/380 ratiometric images were calculated off-line. Some earlier experiments were performed using an alternative imaging system with essentially the same optics but with a TILL polychrome IV monochromator illumination system (TILL Photonics, Planegg, Germany), TILL Imago progressive line-scan digital camera (Scientific Instruments, Madison, WI), and TILL VisiON acquisition and analysis software (see Ref. 7 for detailed description). All experiments were performed at room temperature. The fura-2 fluorescence in mouse parotid acinar cells was calibrated into “estimated” [Ca²⁺⁺], using Equation 1,

\[
[Ca^{2+}] = K_d (R - R_{\text{min}})/(R_{\text{max}} - R)(S_{380}/S_{340})
\]

(Eq. 1)

where \(K_d\) was the fura-2 dissociation constant (150 nM); \(R\) is any given 340/380 ratio value; \(S_{380}/S_{340}\) is the ratio of fluorescence measured for Ca²⁺⁺-free and Ca²⁺⁺-bound fura-2, and \(R_{\text{min}}\) and \(R_{\text{max}}\) are the minimum and maximum ratio values (9). These values were achieved following treatment with 10 μM ionomycin, 0.5 μM carbonyl cyanide 3-chlorophenylhydrazone (to prevent mitochondrial Ca²⁺⁺ uptake), and 30 μM cyclopiazonic acid (CPA) (to prevent Ca²⁺⁺ uptake into the ER) in Ca²⁺⁺-free HEPES-PSS (with 1 mM EGTA) to obtain \(R_{\text{min}}\) values and Ca²⁺⁺ saturated HEPES-PSS (2 mM Ca²⁺⁺) to obtain \(R_{\text{max}}\) values. Average values were obtained from periodic in situ calibration experiments throughout the study and for different microscopes. All experiments were carried out at room temperature (20–22 °C).

**Digital Imaging Fura-2 Fluorescence in Par-C10 Cells**—Initial imaging experiments were performed on Par-C10 cells grown on glass coverslips that formed the base of a gravity-fed perfusion system as described above. Cells were loaded with 5 μM fura-2 in HEPES-PSS for 30 min at room temperature, followed by two washes with HEPES-PSS, and a further 30 min de-esterification period in serum-free media at 37 °C. Under these conditions cells were imaged as described above using a ×40 oil immersion objective with 3 × 3 binning and 1-Hz acquisition rate. In experiments where Par-C10 cells were grown on Transwell supports, the loading conditions, perfusion system, microscope optics, and image acquisition parameters were altered to maximize fluorescence capture and detection. First, cells were loaded with 5 μM fura-2 in HEPES-PSS for 60 min at 37 °C in a bicarbonate-buffered PSS, containing (in mM) 116 NaCl, 5 KCl, 1 CaCl₂, 1 MgSO₄, 2.8 Na-HEPES, 2.2 HEPES, 10 glucose, and 25 NaHCO₃, and followed by a further 30-min de-esterification period in serum-free media at 37 °C. All solutions contained 2 mM probenacid throughout to inhibit rapid de-esterification period in serum-free media at 37 °C. All solutions contained 2 mM probenacid throughout to inhibit rapid de-esterification period. Following dye loading, each side (apical or basolateral) of the cell monolayer was separately perfused using a modified chamber connected to two separate gravity-fed perfusion systems similar to that described above. The modified chamber consisted of a Transwell insert that sat in its corresponding well with its base cut out, which effectively acted as a collar to support the insert, and together these were placed on top of the standard perfusion chamber described above. The collar was engineered so that the polyester filter was 2–3 mm from the glass coverslip, which allowed efficient perfusion of the basolateral side of the monolayer. The apical side of the cells was perfused using the base of a 50-ml Falcon tube that had been cut, inverted, and placed over the Transwell insert with two needles inserted that served as inlet and outlet tubes. The perfusion system was engineered to allow the efficient laminar flow perfusion of both sides of the Par-C10 monolayer, as assessed by separate perfusion with carbachol (CCh) and ATP (see “Results”). Fluorescence imaging experiments were performed using the same microscope described above with a ×40 extra long working distance objective. This was because the focal plane of the cells was some distance above the basolateral perfusate layer. However, the extra long working distance objective (NA, 0.6) transmitted significantly less light than the ×40 S-Fluor objective (NA, 1.3) used above. This dramatically reduced the total fluorescence that could be captured by the lens; therefore, images were acquired with 8 × 8 binning to improve the signal-to-noise. Background subtraction was
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achieved off line following perfusion of the cells with distilled water to remove the cells from the Transwell support.

Western Blotting and Phosphorylation of PMCA—All methods pertaining to the immunodetection and phosphorylation of PMCA proteins were similar to previous studies (6, 7), except specific antibodies directed against PMCA1–4 were used in addition to the nonspecific antibody (5F10). All PMCA antibodies were obtained from Affinity Bioreagents (Golden, CO). For Western blotting studies parotid acinar cells were prepared as described above, rapidly centrifuged, and resuspended in ice-cold lysis buffer containing (in mM) 50 Tris-HCl (pH 7.4), 250 NaCl, 5 EDTA, 100 NaF, 1% Triton-X-100 and EDTA-free Complete protease inhibitor mixture tablets (Roche Diagnostics). Protein samples were prepared as described previously (6, 7). Different amounts (10, 30, and 50 µg) of parotid acinar cell protein were loaded onto the same gel. For the PMCA3 and PMCA4, where detection of visible bands was either absent or low, protein samples from mouse brain were run on the same gel as a positive control. Brain lysates were prepared by rapidly dissecting the brain followed by homogenization in ice-cold lysis buffer using a glass Dounce homogenizer. For the phosphorylation assay, parotid acinar cells were isolated from 2 to 4 mice, appropriately aliquoted, and treated for 10 min with or without 1 µM CCh and 10 µM forskolin, which was found to cause maximum phosphorylation of the PMCA (7). Cell lysates were then sonicated and left on ice to solubilize for 30 min, followed by centrifugation at 300 × g to remove insoluble protein and cell debris (7). Each lysate was incubated with 80 µl of protein-A/G-agarose beads (Pierce) for 1 h at 4 °C to preclariy any nonspecific binding. Following centrifugation, supernatants were incubated with the appropriate PMCA antibody (~1 µg/mg protein; PMCA1–4, Affinity Bioreagents, Golden, CO) for 1 h, followed by 80 µl of protein-A/G-agarose beads for a further hour at 4 °C, to immunoprecipitate the corresponding PMCA protein. As a secondary control (blank), an aliquot of cell lysates from untreated cells was incubated with beads but without any antibodies. Immunoprecipitated proteins were separated by SDS-PAGE (7.5%) and Western-blotted using the phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology) to detect phosphorylated PMCA protein.

Co-immunoprecipitation of Ezrin, EBP50, NHERF-2, and PKA-RIIβ—In some experiments, parotid acinar cell lysates were incubated with monoclonal antibodies against either ezrin, EBP50, the regulatory subunit of PKA (RIIβ) (Transduction Laboratories), or the polyclonal antibody against the Na+/H+ exchanger regulatory factor-2 (NHERF-2) (Alpha Diagnostic International Inc, San Antonio, TX). Immunoprecipitated protein was prepared as described above, separated by SDS-PAGE alongside cell lysates, and Western-blotted using the nonspecific PMCA antibody (5F10, Affinity Bioreagents). PKA-RIIβ immunoprecipitates were also Western-blotted with the affinity-purified polyclonal antibody (CT-2) directed against the extreme C-terminal 17 amino acids (GFLGSNTPHENHHPMPHP) of the type 2 IP3R (IP3R-2). This was a kind gift from Dr. David Yule (University of Rochester Medical Center, Rochester, NY), originally made by Pocono Rabbit Farms (Canadensis, PA).

Preparation of Mouse Parotid Tissue Sections—Mouse parotid glands were dissected, immersed in Tissue-Tek embedding medium (Sakura Finetek Europe), and immediately snap-frozen in liquid nitrogen. Tissue sections (8–10 µm) were cut using a Cryostat (Leica CM3050), mounted onto Superfrost slides (VWR Scientific), and methanol-fixed at −20 °C for 10 min. Methanol was removed by washing twice with phosphate-buffered saline (PBS) and kept at −20 °C until ready for use.

Immunofluorescence of Mouse Parotid Tissue Sections—Tissue sections were incubated with 50 mm glycine for 10 min at room temperature, permeabilized for 10 min in PBS containing 0.3% Triton X-100, and washed in PBS before blocking with blocking solution containing PBS with 5% normal goat serum (Jackson ImmunoResearch), 1% BSA (Sigma), 0.1% gelatin (Sigma). Tissue sections were incubated with the primary antibodies in PBS and 1% BSA overnight at 4 °C; PMCA1 (polyclonal, 1:100 dilution), PMCA2 (polyclonal, 1:100 dilution), and PMCA4 (monoclonal, 1:100) were all from Affinity Bioreagents (Golden, CO), and ezrin and EBP50 (monoclonal, 1:100 dilution) were from BD Transduction Laboratories. After the primary antibody incubations, sections were washed in PBS and 1% BSA three times for 10 min, prior to incubation with AlexaFluor488-conjugated secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature and goat anti-mouse (for monoclonal primary antibodies) and goat anti-rabbit (for polyclonal primary antibodies).

Immunofluorescence of Acutely Isolated Mouse Parotid Acinar Cells—Following isolation, equal aliquots of cells were centrifuged and resuspended in HEPES-PSS with or without CCh (1 µM) and/or forskolin (10 µM) for 10 min at room temperature. Following treatment, cells were fixed and permeabilized with ice-cold methanol for 30 min at −20 °C. The cells were washed three times for 10 min in PBS followed by a 10-min incubation with 50 mm glycine at room temperature before blocking for 1 h with blocking solution. Cells were incubated with primary antibodies in blocking solution overnight at 4 °C in a humidity chamber as follows: PKA-RIIβ (monoclonal, 1:300 dilution; BD Transduction Laboratories) and CT-IP3R-2 (polyclonal, 1:20 dilution). After the primary antibody incubations, cells were washed in blocking solution three times for 10 min prior to incubation with secondary antibodies for 1 h at room temperature in the dark (AlexaFluor488-conjugated goat anti-mouse, 1:500 dilution; AlexaFluor568-conjugated goat anti-rabbit, 1:200 dilution). Following incubation with secondary antibodies, cells were washed for an additional two times for 10 min prior to a 5-min incubation with 4′,6-diamidino-2-phenylindole for staining of the nuclei.

In the absence of blocking peptides, nonspecific binding of each fluorescent secondary antibody was determined by incubating some tissue sections or cells without primary antibody (negative control). Fluorescent images were acquired using either an inverted Leica SP2 or SP5 AOBS (filter-free) laser scanning confocal microscope with a ×40 or ×63 objective. Control slides (incubated with the appropriate secondary antibody only) were imaged first, and the microscope settings (gain and offset) were adjusted so that minimum background fluorescence was detected from nonspecific binding of the secondary antibodies. With the microscope settings the same, images
PKA-mediated phosphorylation of the PMCA (7). This study utilized a simple assay to measure \([\text{Ca}^{2+}]_{i}\) clearance that was due primarily to PMCA activity. This involved treating cells with 30 \(\mu\text{M}\) CPA that slowly raised \([\text{Ca}^{2+}]_{i}\), because of leak from the ER and activation of store-operated \(\text{Ca}^{2+}\) entry (10, 11). The increase in \([\text{Ca}^{2+}]_{i}\), reached a peak and then slowly declined to a new steady state, presumably because of a balance of \(\text{Ca}^{2+}\) efflux and \(\text{Ca}^{2+}\) influx. Removal of external \(\text{Ca}^{2+}\) ([\(\text{Ca}^{2+}]_{0}\)), by chelation with 1 \(\text{mM}\) EGTA, evoked an immediate clearance of \([\text{Ca}^{2+}]_{i}\), that was primarily due to the PMCA activity (7) (Fig. 1A). The rate of \([\text{Ca}^{2+}]_{i}\) clearance was quantified by fitting to a single exponential decay, and the time constants \((\tau)\) were compared between control and forskolin-treated cells (7). Under these conditions forskolin caused an \(\sim 2\)-fold increase in the rate of \([\text{Ca}^{2+}]_{i}\) clearance (7) (Fig. 1C). We repeated these experiments, except we also initiated the measurement of clearance by removing \([\text{Ca}^{2+}]_{i}\), at the peak of the CPA-evoked \([\text{Ca}^{2+}]_{i}\), response (Fig. 1B, “Peak” response), and similar to above, the clearance rate was compared between control and forskolin-treated cells (Fig. 1, B and D). However, under these conditions forskolin had no significant effect on the rate of \([\text{Ca}^{2+}]_{i}\) clearance (control cells, \(\tau = 49 \pm 2\), \(n = 15\) cells; forskolin-treated cells, \(\tau = 44 \pm 2\), \(n = 20\) cells) compared with when measurement of clearance was initiated at the reduced elevated steady state \([\text{Ca}^{2+}]_{i}\), level (Fig. 1A, “Plateau” response; control cells, \(\tau = 44 \pm 3\), \(n = 21\) cells; forskolin-treated cells, \(\tau = 28 \pm 2\), \(n = 19\) cells). One possible explanation for the difference is that the ER \(\text{Ca}^{2+}\) leak is greater during the “peak” response compared with the “plateau” response, thereby slowing the measured \([\text{Ca}^{2+}]_{i}\) clearance and “apparent” PMCA activity, which may mask any effect of forskolin. However, the control \([\text{Ca}^{2+}]_{i}\) clearance for both experimental paradigms (“plateau”, Fig. 1A versus “peak”, Fig. 1B) was not significantly different (“plateau”, \(\tau = 44 \pm 3\); “peak” \(\tau = 49 \pm 2\)). Another possibility is the effect of forskolin is time-dependent, because during the “plateau” response (Fig. 1A) the cells were exposed to forskolin for a longer period of time (\(\sim 3\)–4 min to allow for the return to a new steady state \([\text{Ca}^{2+}]_{i}\)). Furthermore, after closer examination of the data, it was noticed that there was a spatial difference in the clearance. This was further re-analyzed by comparing the apical (shown as the red boxes and traces in Fig. 2) with the basolateral clearance (shown as the blue boxes and traces in Fig. 2) for the peak response in control (Fig. 2A) and forskolin-treated cells (Fig. 2B). On average in untreated control cells there was no significant difference in

were then acquired from slides that had been incubated with both primary antibody and corresponding secondary antibody. Therefore, the fluorescence detected represents fluorescence above background fluorescence caused by nonspecific binding of the secondary antibody. Additionally, for dual labeling the range for the 488 and 568 nm lasers was limited to ensure no overlap in excitation. Fluorescent images were acquired sequentially frame by frame with 488 and 568 nm lasers and UV light. Digitized images were processed using Jasc PhotoShop Pro software.

Data Analysis and Experimental Design—For comparisons of \([\text{Ca}^{2+}]_{i}\) clearance rates, different analytical approaches were utilized that are described in more detail under “Results.” In experiments using mouse parotid acinar cells, \([\text{Ca}^{2+}]_{i}\), clearance was fitted to a single exponential decay to yield the time constant \((\tau)\) as a measure of rate. In experiments using Par-C10 cells, \([\text{Ca}^{2+}]_{i}\), clearance was initiated at a reduced plateau \((\tau, p < 0.05, n = 5\) experiments) (C) but not at the “peak” \([\text{Ca}^{2+}]_{i}\), response (D). There was also no significant difference in \([\text{Ca}^{2+}]_{i}\), clearance rate of untreated control cells when the measurement of clearance was initiated at the plateau response (A) compared with the peak response (B).

RESULTS

Forskolin Differentially Potentiates Apical \([\text{Ca}^{2+}]_{i}\), Clearance in Acutely Isolated Parotid Acinar Cells—It has been shown previously in mouse parotid acinar cells that activation of PKA potentiated \([\text{Ca}^{2+}]_{i}\), clearance because of a \(\text{Ca}^{2+}\)-dependent, steady state plateau (Fig. 1). In experiments using Par-C10 cells, \([\text{Ca}^{2+}]_{i}\), between different regions of the same cell (paired Student’s test) (C) but not at the “peak” \([\text{Ca}^{2+}]_{i}\), response (D). There was also no significant difference in \([\text{Ca}^{2+}]_{i}\), clearance rate of untreated control cells when the measurement of clearance was initiated at the plateau response (A) compared with the peak response (B).
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**FIGURE 2. PKA activation differentially potentiates apical \([\text{Ca}^{2+}]\) clearance in mouse parotid acinar cells.** Representative traces and corresponding pseudocolor-enhanced ratiometric images showing the differential effect of 10 \(\mu M\) forskolin treatment (B) on apical (red box and trace) \([\text{Ca}^{2+}]\), clearance compared with control cells (A). The measurement of \([\text{Ca}^{2+}]\), clearance was initiated by removal of external \(\text{Ca}^{2+}\) following treatment with CPA at the peak \([\text{Ca}^{2+}]\), response. \([\text{Ca}^{2+}]\), clearance rate was fitted to a single exponential decay to yield the time constant (\(\tau\)), and comparisons of apical and basolateral regions were made using a paired \(t\) test (inset represents the mean time constants at the apical and basolateral region of cells; *, \(p < 0.001\), \(n = 5\) experiments). C and D, summary of mean \([\text{Ca}^{2+}]\), clearance data comparing local \([\text{Ca}^{2+}]\), clearance when the measurement of \([\text{Ca}^{2+}]\), clearance was initiated at the peak (C) or the plateau (D) of the CPA-evoked \([\text{Ca}^{2+}]\), response. When clearance was initiated at the peak \([\text{Ca}^{2+}]\), response forskolin differentially increased the rate of PMCA activity in the apical region (*, \(p < 0.001\) forskolin, apical versus basal, paired \(t\) test, C) but failed to have any significant effect on the basal region of parotid acinar cells. However, this was not the case when clearance was initiated at the reduced plateau (D), because apical and basal clearance were both similarly affected by forskolin (*, \(p < 0.05\), forskolin versus control, unpaired \(t\) test).

Clearance rates between the apical and basolateral regions (Fig. 2A, apical \(\tau = 49 \pm 3\); basolateral \(\tau = 48 \pm 2\), \(n = 5\), 19 cells, as assessed by a paired \(t\) test). However, in forskolin-treated cells, the apical clearance rate was significantly faster (\(\tau = 32 \pm 2\)) than the basolateral clearance (\(\tau = 55 \pm 2\), \(n = 5\), 20 cells, \(p < 0.001\), as assessed by a paired \(t\) test; Fig. 2B). Furthermore, in forskolin-treated cells the basolateral clearance was not significantly different from the basolateral clearance in untreated control cells (control \(\tau = 48 \pm 2\) versus forskolin \(\tau = 55 \pm 2\), as assessed by an unpaired \(t\) test). These data suggest that the apical PMCA is differentially potentiated by PKA activation. Moreover, with the “plateau” response (see Fig. 1A), whereby cells were exposed to forskolin for a longer period of time, both apical and basolateral \([\text{Ca}^{2+}]\), clearance rates were significantly potentiated in forskolin-treated cells compared with untreated control cells (see summary of mean \([\text{Ca}^{2+}]\), clearance data, Fig. 2, C and D). This therefore suggests that the basolateral PMCA has the capacity to be potentiated by PKA activation. Moreover, this helps to explain why forskolin treatment had no significant effect on the average “global” \([\text{Ca}^{2+}]\), clearance with the peak response (Fig. 1B), yet significantly increased the global clearance with the plateau response (Fig. 1A, and see summary of mean \([\text{Ca}^{2+}]\), clearance data, Fig. 2, C and D). It was also noticed that in forskolin-treated cells the rate of increase of the CPA-evoked \([\text{Ca}^{2+}]\), response appeared slower in the apical region compared with the basal region (Fig. 2B). This was analyzed by comparing the time-to-peak of the CPA-evoked \([\text{Ca}^{2+}]\), response in the apical and basal regions of forskolin-treated and control cells. Using an unpaired analysis, the basal time-to-peak was not significantly different between forskolin-treated (255 ± 21 s, \(n = 5\), 19 cells) and control cells (254 ± 20 s, \(n = 5\), 21 cells). The apical time-to-peak appeared slower in forskolin-treated cells (308 ± 15 s) compared with control cells (254 ± 20 s); however, this did not reach significance (*, \(p = 0.07\), unpaired \(t\) test). Nevertheless, using a paired analysis, the apical time-to-peak was consistently slower than the corresponding basal region in forskolin-treated cells (*, \(p = 0.02\), paired \(t\) test). This is consistent with a potentiation of the apical \([\text{Ca}^{2+}]\), clearance. Collectively, these data suggest that
PKA activation leads to a more efficient regulation of the apical PMCA compared with the basolateral PMCA.

**PKA Activation Potentiates \([\mathrm{Ca}^{2+}]\), Signaling in Par-C10 Cells**—To further test the hypothesis that PKA differentially potentiates the apical PMCA activity, we adopted a more direct approach using a model in which the apical and basolateral PMCA activity could be functionally isolated. It was first necessary to confirm that Par-C10 cells exhibit similar \([\mathrm{Ca}^{2+}]\) signaling properties to mouse parotid acinar cells (7). Consistent with observations in mouse parotid acinar cells (7), forskolin markedly potentiated CCh-evoked \([\mathrm{Ca}^{2+}]\), signaling in Par-C10 cells grown on glass coverslips (Fig. 3A). Specifically, both the amplitude and frequency of CCh-evoked \([\mathrm{Ca}^{2+}]\), oscillations were found to be enhanced by forskolin, suggesting that both \(\mathrm{Ca}^{2+}\) release and \([\mathrm{Ca}^{2+}]\), clearance may be potentiated (Fig. 3A). However, it was noticed that Par-C10 cells were much less responsive to CCh (100 \(\mu\)M, Fig. 3) compared with mouse parotid acinar cells (0.1–1 \(\mu\)M) (7), suggesting that these cells may have undergone some de-differentiation resulting in loss of muscarinic receptors. Nevertheless, these data suggest that the \([\mathrm{Ca}^{2+}]\), signaling machinery in Par-C10 cells exhibits similar properties to mouse parotid acinar cells, making them a useful comparative model.

**CCh and ATP Increase \([\mathrm{Ca}^{2+}]\), When Applied to Opposite Sides of Par-C10 Cells Grown on Transwell Supports**—We next wanted to test and validate that the apical and basolateral sides of the Par-C10 monolayer could be separately perfused. Par-C10 cells were grown on Transwell supports for a minimum of 10 days thereby allowing the differentiation and formation of a tight epithelial monolayer (see “Experimental Procedures”). The TEER was routinely measured and was found to be 1000–3000 ohm-cm\(^2\), consistent with the formation of a tight epithelium (8). This was further confirmed by perfusing either side of the monolayer with CCh and/or ATP, which is known to activate muscarinic receptors on the basolateral membrane and purinergic receptors on the apical membrane, respectively (Fig. 3B) (8). Consistent with these studies, it was found that 100 \(\mu\)M CCh failed to increase \([\mathrm{Ca}^{2+}]\), when applied to the apical side but evoked a robust increase in \([\mathrm{Ca}^{2+}]\), when applied to the basolateral side (Fig. 3B, \(n = 4\), 152 cells). Conversely, 10 \(\mu\)M ATP increased \([\mathrm{Ca}^{2+}]\), when applied to the apical side but failed to evoke any response when applied to the basolateral side (Fig. 3B, \(n = 4\), 152 cells). These data confirm that the apical and basolateral sides of the Par-C10 cell monolayer can be separately perfused to generate functionally distinct responses.

**Functional Separation of Apical and Basolateral PMCA Activity in Par-C10 Cells**—We next utilized this property of the Par-C10 cells by separately perfusing the apical and basolateral sides of the Par-C10 cell monolayer with the PMCA inhibitor \(\mathrm{La}^{3+}\) (1 mM). This allowed the functional isolation of the apical and basolateral PMCA activity during the typical \([\mathrm{Ca}^{2+}]\), clearance assays described in Figs. 1 and 2 (Fig. 4). At this concentration (1 mM), \(\mathrm{La}^{3+}\)
inhibits both Ca\(^{2+}\) influx and Ca\(^{2+}\) efflux, effectively “sealing” the cell and thereby trapping the Ca\(^{2+}\) so that it can neither enter nor leave the cell (12, 13). This was confirmed by the addition of La\(^{3+}\) to both sides of the monolayer, which almost completely prevented any clearance of [Ca\(^{2+}\)]\(_i\) following the removal of external Ca\(^{2+}\) (Fig. 4A and mean data in Fig. 5B). However, removal of La\(^{3+}\) using EGTA, which binds La\(^{3+}\) with high affinity, slowly reversed the inhibition of the PMCA, which then started to rapidly clear Ca\(^{2+}\) from the cytosol (Fig. 4A). The re-addition of [Ca\(^{2+}\)]\(_i\) increased [Ca\(^{2+}\)]\(_i\), presumably because of the activation of store-operated Ca\(^{2+}\) entry, and the subsequent addition of La\(^{3+}\) to the basolateral side of the cells slowed but did not completely stop the [Ca\(^{2+}\)]\(_i\) clearance. This slowed [Ca\(^{2+}\)]\(_i\) clearance was due to Ca\(^{2+}\) being “forced” to be transported by the only available route, across the apical membrane, suggesting that the apical PMCA activity was functionally isolated. The schematic in Fig. 4B illustrates the different experimental conditions as follows: in the absence of La\(^{3+}\) (Fig. 4B, panel i; total clearance), when La\(^{3+}\) inhibits PMCA activity on both sides (Fig. 4B, panel ii; “residual” clearance), when La\(^{3+}\) is applied to the basolateral side (Fig. 4B, panel iii; apical PMCA functionally isolated), and when La\(^{3+}\) is applied to the apical side (Fig. 4B, panel iv; basolateral PMCA functionally isolated).

PKA Activation Differentially Potentiates Apical PMCA Activity in Par-C10 Cells—The above technique was therefore used to functionally isolate either the apical or basolateral PMCA activity, by applying La\(^{3+}\) to the opposite side, in the absence and presence of forskolin (summarized in Fig. 5). Quantification of clearance rates under these different conditions was more difficult (compared with Figs. 1–3) for the following reasons. First, in situ calibration experiments to convert 340/380 ratio values into [Ca\(^{2+}\)]\(_i\) were unsuccessful because perfusion of the cells with calibration solutions (ionomycin, carbonyl cyanide 4 trifluoromethoxyphenylhydrazone, CPA; see “Experimental Procedures”) caused many of the cells to

![FIGURE 5. PKA activation differentially potentiates apical PMCA activity when functionally isolated in Par-C10 cells.](image-url)
either lyse or wash off the Transwell membrane. This made comparisons of clearance rates difficult to analyze, especially if there were differences in preclearance starting $[\text{Ca}^{2+}]_i$, from which clearance was measured. Therefore, clearance was normalized to the same starting ratio value for each cell (see inset for each representative trace, Fig. 5, A and C–F). Second, under conditions where the $[\text{Ca}^{2+}]_i$ clearance decreased to such an extent, for example when the basolateral PMCA was functionally isolated, the clearance could not be accurately fitted to a single exponential decay and rather followed a linear relationship. Therefore, to include all of the data in the analysis, clearance was fitted to a linear regression over a 30-s period, expressed as change in ratio per min ($R_{\text{min}}$). Finally, there was a large degree of variability in clearance between cells both within the same experiment and between experiments. This was likely due to differences in dye loading combined with low levels of fluorescence detection because of the extra long working distance objective. This means that any change in clearance caused by a particular treatment/maneuver may be masked by this large variability; therefore, a paired experimental design was utilized. Time-matched control experiments were performed whereby two successive $[\text{Ca}^{2+}]_i$ clearance phases were initiated by the removal of $[\text{Ca}^{2+}]_o$ in the presence of CPA (Fig. 5A Time-matched control). These experiments showed that the second $[\text{Ca}^{2+}]_i$ clearance rate ($R_{\text{Tot1}}$, $\tau = 51 \pm 3$, Fig. 5A) was not significantly different from the first $[\text{Ca}^{2+}]_i$ clearance rate ($R_{\text{Tot2}}$, $\tau = 48 \pm 4$, Fig. 5A). This suggests that the passive ER Ca$^{2+}$ leak does not contribute more to the first clearance phase as one would expect this to be much slower than the second clearance phase if the stores have not been sufficiently depleted. This was an important observation as it also means that La$^{3+}$ could be applied during the second clearance phase to isolate either the apical ($R_{\text{Apical}}$) or basolateral PMCA ($R_{\text{Baso}}$), and the clearance was normalized to the total clearance ($R_{\text{Tot}}$) and expressed as a percentage (Fig. 5B, mean data). This was achieved by measuring the linear clearance rate at the same starting ratio (see dashed box Fig. 5, A and C–F). This therefore provides a convenient means of quantifying the functional activity of either the apical or basolateral PMCA in the absence (Fig. 5, C and E) and presence (Fig. 5, D and F) of forskolin.

**FIGURE 6.** Further validation that the measured $[\text{Ca}^{2+}]_i$ clearance is due to PMCA activity. The measurement of $[\text{Ca}^{2+}]_i$ clearance was initiated by the removal of external Ca$^{2+}$ ([Ca$^{2+}]_o$) at the peak of the CPA-evoked $[\text{Ca}^{2+}]_i$ response and fitted to a single exponential decay to yield the time constant ($\tau$) in untreated mouse parotid acinar cells (A, panel i) and Par-C10 cells (B, panel i). To test the contribution of mitochondrial Ca$^{2+}$ uptake to the measured $[\text{Ca}^{2+}]_i$ clearance, mouse parotid acinar cells (A, panel ii) and Par-C10 cells (B, panel ii) were preincubated with 10 $\mu$M Ru360 for 30 min. To test the contribution of Na$^+$/Ca$^{2+}$ exchange to the measured $[\text{Ca}^{2+}]_i$ clearance, all Na$^+$ ions were replaced with NMDG in all solutions, and clearance was measured in mouse parotid acinar cells (A, panel iii) and Par-C10 cells (B, panel iii). Mouse parotid acinar cells (A, panel iv) and Par-C10 cells (B, panel iv) were preincubated with increasing concentrations (10 or 30 $\mu$M) of the specific PMCA inhibitor, 5-(and-6)-carboxy eosin diacetate succinimidyl ester (carboxy eosin) for increasing amounts of time (10 or 30 min) prior to the initiation of the $[\text{Ca}^{2+}]_i$ clearance assay.
PKA Regulation of the Apical PMCA (mean data summarized in Fig. 5B). Consequently, during time-matched control experiments where La\(^{3+}\) was absent, the second clearance phase \((R_{\text{tot2}})\) was on average 104 ± 7% \((n = 3, 68\) cells) of the first clearance phase \((R_{\text{tot1}})\) and thus represented the “maximum PMCA activity.” Similar analysis was carried out where La\(^{3+}\) was applied to both sides of the cells to block all PMCA activity (as shown in Fig. 4A). This residual clearance was 5 ± 1% \((n = 4, 49\) cells) of the total clearance \((R_{\text{tot1}})\), which was still yet significant \((p < 0.05,\) one sample t test) and thus represented the minimum PMCA activity. Nevertheless, this residual clearance was significantly lower than the maximum PMCA activity when no La\(^{3+}\) was present \((p < 0.001,\) Mann-Whitney test). In untreated control cells the apical PMCA was 39 ± 4% \((n = 6, 148\) cells; see Fig. 5, C and B) of the total clearance, which was significantly lower than the maximum PMCA activity \((*, p < 0.001,\) Mann-Whitney test) but significantly higher than the residual clearance \((**, p < 0.001,\) Mann-Whitney test; Fig. 4A and mean data Fig. 5B). Likewise, the basolateral PMCA was 20 ± 4% \((n = 6, 127\) cells; see Fig. 5, E and B) of the total clearance, which was also significantly lower than the maximum PMCA activity \((*, p < 0.001,\) Mann-Whitney test; Fig. 5A and mean data Fig. 5B) yet significantly higher than the residual clearance \((**, p < 0.005,\) Mann-Whitney test Fig. 4A and mean data Fig. 5B). This therefore provides quantitative evidence that the apical and basolateral PMCA activities have been successfully separated functionally. Moreover, these data revealed that the basolateral PMCA activity was significantly lower than the apical PMCA activity \((p < 0.01,\) Mann-Whitney test, Fig. 5, C and E, and mean data Fig. 5B). More importantly, however, in forskolin-treated cells the apical PMCA activity was markedly potentiated to 149 ± 9% \((n = 4, 76\) cells; see Fig. 5, D and B; †, \(p < 0.001\)) of the total clearance, whereas the basolateral PMCA activity was largely unaffected \((19 ± 3\% n = 4, 88\) cells; see Fig. 5, F and B). This further supports the original conclusion that the apical PMCA is differentially potentiated by PKA activation.

Further Validation That the Experimental [Ca\(^{2+}\)] Clearance Is Due to the PMCA Activity—Because of the residual [Ca\(^{2+}\)], clearance following addition of La\(^{3+}\) to both sides of Par-C10 cells and because of the nonspecific nature of La\(^{3+}\), it was necessary to further verify that the measured [Ca\(^{2+}\)] clearance was due to the PMCA activity. Other possible mechanisms for [Ca\(^{2+}\)] clearance under these conditions include mitochondrial Ca\(^{2+}\) uptake and Na\(^{+}\)-Ca\(^{2+}\) exchange (NCX). First, mitochondrial Ca\(^{2+}\) uptake was tested by preincubating cells with 10 \(\mu M\) Ru360 for 30 min prior to beginning the [Ca\(^{2+}\)] clearance assay (Fig. 6, A, panel ii, and B, panel ii). This has been shown previously to inhibit mitochondrial Ca\(^{2+}\) uptake in mouse parotid acinar cells (14). The contribution of the NCX activity to the measured [Ca\(^{2+}\)] clearance was also tested by replacing all external Na\(^{+}\) ions (138 mm) with N-methyl-D-glucamine (NMDG) in all solutions (Fig. 6, A, panel iii, and B, panel iii). Under both of these conditions, [Ca\(^{2+}\)] clearance was not significantly different from control mouse parotid acinar cells (Fig. 6A, panel i, control \(\tau = 45 ± 2\%, n = 6, 66\) cells; Fig. 6A, panel ii, Ru360 \(\tau = 46 ± 2\%, n = 4, 54\) cells; Fig. 6A, panel iii, NMDG \(\tau = 47 ± 3\%, n = 4, 48\) cells) or Par-C10 cells (Fig. 6B, panel i, control \(\tau = 58 ± 9\%, n = 5, 83\) cells; Fig. 6B, panel ii, Ru360 \(\tau = 63 ± 8\%, n = 4, 62\) cells; Fig. 6B, panel iii, NMDG \(\tau = 64 ± 5\%, n = 5, 96\) cells). Further validation was achieved by preincubating cells with the more specific inhibitor, carboxyoxin (15, 16). Because this binds to the cytosolic side of the PMCA, it was loaded into the cells as the 5-(and-6)-carboxyoxsin diacetate succinimidyl ester similar to the fluorescent dye fura-2 acetoxymethyl ester. Mouse parotid acinar cells were loaded under three different conditions in an attempt to progressively increase the intracellular concentration of carboxyoxsin. These were as follows: 10 \(\mu M\) for 10 min, 10 \(\mu M\) for 30 min, and 30 \(\mu M\) for 30 min (Fig. 6A, panel iv, and B, panel iv). In mouse parotid acinar cells there was very clear concentration-dependent inhibition of [Ca\(^{2+}\)] clearance under each of the three loading conditions (Fig. 6A, panel iv, 10 \(\mu M/10\) min \(\tau = 105 ± 4\%, n = 4, 28\) cells; 10 \(\mu M/30\) min \(\tau = 164 ± 10\%, n = 4, 34\) cells; 30 \(\mu M/30\) min \(\tau = 276 ± 40\%, n = 4, 19\) cells; *, \(p < 0.01\)). It was also noticed that the resting [Ca\(^{2+}\)], prior to the addition of CPA progressively increased with increasing concentrations of carboxyoxsin (Fig. 6, A, panel iv, and B, panel iv). Similar results were obtained with Par-C10 cells (Fig. 6B, panel iv, 10 \(\mu M/10\) min \(\tau = 239 ± 26\%, n = 3, 53\) cells; 10 \(\mu M/30\) min \(\tau = 350 ± 59\%, n = 3, 42\) cells; *, \(p < 0.01\)). The highest concentration of carboxyoxsin caused the Par-C10 cells to be completely unresponsive to CPA, suggesting that the cells had died. Collectively, these data clearly demonstrate that the measured [Ca\(^{2+}\)] clearance is due to the PMCA activity further validating the data in Fig. 1, 2, 4, and 5.

Expression and Localization of Specific PMCA Isoforms in Mouse Parotid Acinar Cells—Western blotting and immunofluorescence experiments were carried out, using specific anti-
bodies, to determine expression and localization of PMCA1–4 in mouse parotid acinar cells. Western blotting revealed that PMCA1 (Fig. 7B), PMCA2 (Fig. 7C), and PMCA4 (Fig. 7E) were all expressed in parotid acinar cells. PMCA4 was detected in low abundance relative to mouse brain lysates (see long exposure, Fig. 7E). PMCA3, which was detected in brain, could not be detected in mouse parotid acinar cells (Fig. 7D).

Immunofluorescence experiments were performed whereby fluorescent images (pseudo-colored green) were superimposed over the corresponding bright field image so that the localization of specific proteins could be more accurately determined (Fig. 8A). These data revealed that PMCA1 was localized to all regions of the plasma membrane; PMCA2 was localized to the basolateral membrane, and PMCA4 exhibited an almost exclusive apical localization (Fig. 8A, panel i). Furthermore, the adaptor proteins, ezrin (Fig. 8A, panel iii) and EBP50 (Fig. 8A, panel iv), were also found to exhibit a predominantly apical distribution. In the absence of suitable blocking peptides, the corresponding fluorescence images of parotid tissue sections stained with the AlexaFluor488-conjugated secondary antibodies alone (goat anti-mouse and goat anti-rabbit) are also shown in Fig. 8A, panel ii, as a negative control. These images were acquired using the same offset and gain settings as images shown in Fig. 8A, panels i, iii and iv, suggesting that the fluorescence is above any fluorescence caused by nonspecific binding of the secondary antibodies.

In Situ Phosphorylation of PMCA1—Previous in situ phosphorylation assays in mouse parotid acinar cells have shown that the combined treatment with CCh and forskolin causes a Ca\(^{2+}\)-dependent, PKA-mediated phosphorylation of the PMCA1 (7). These experiments were performed by immunoprecipitating with the anti-PMCA1 antibody and Western blotting using the phospho-(Ser/Thr) substrate antibody to detect phosphorylated PMCA1. C, parotid cell lysates (from four mouse parotids) were immunoprecipitated with antibodies (AB) for PKA-RII\(\beta\), ezrin, EBP50, and PMCA (5F10), separated by SDS-PAGE (7% gel), and subsequently Western-blotted with the IP3R-2 antibody (anti-CT-2).

**FIGURE 8.** Biochemical identification of PMCA, ezrin, and EBP50 localization, PMCA phosphorylation, and PKA-RII\(\beta\) binding partners in parotid acinar cells. A, immunofluorescence experiments of methanol-fixed mouse parotid gland tissue slices (8–10 \(\mu\)m) showing the localization of PMCA1, PMCA2, PMCA4 (A, panel i), ezrin (A, panel iii), and EBP50 (A, panel iv). Pseudocolor-enhanced fluorescent images were superimposed over the corresponding bright field images for clarity. As a negative control, tissue sections were incubated without any primary antibody but with the appropriate fluorescent secondary antibody alone (FL-goat anti-mouse or FL-goat anti-rabbit) (A, panel ii). Confocal microscope settings were the same. B, representative experiment of an in situ phosphorylation assay following treatment with (+) or without (−) 1 \(\mu\)M CCh and 10 \(\mu\)M forskolin (Forsk). Parotid lysates were immunoprecipitated (IP) with the anti-PMCA1 antibody and Western blotted (WB) using the phospho-(Ser/Thr) substrate antibody to detect phosphorylated PMCA1. C, parotid cell lysates (from four mouse parotids) were immunoprecipitated with antibodies (AB) for PKA-RII\(\beta\), ezrin, EBP50, and PMCA (5F10), separated by SDS-PAGE (7% gel), and subsequently Western-blotted with the nonspecific PMCA antibody (5F10). D, parotid cell lysates (from four mouse parotids) were immunoprecipitated with antibodies for PKA-RII\(\beta\) and subsequently Western-blotted with the IP, R-2 antibody (anti-CT-2).
cient regulation of PMCA by PKA. To test this hypothesis, PKA was immunoprecipitated from parotid lysates using an antibody against the regulatory subunit of PKA (PKA-RIIβ) followed by the subsequent Western blotting with the PMCA antibody (5F10). These experiments were performed following the combined treatment of cells with or without CCh and forskolin, as this may facilitate the targeting of PKA to the PMCA because maximum phosphorylation was observed under these conditions (7) (Fig. 8B). In addition, because the adaptor proteins, ezrin and EBP50, were shown to be apically distributed in mouse parotid acinar cells (Fig. 8A, panels iii and iv), we reasoned that these proteins may also contribute to targeting PKA to the PMCA under similar conditions. However, these experiments proved unsuccessful as no PMCA protein was reproducibly detected in any PKA-RIIβ, ezrin, or EBP50 immunoprecipitates (see Fig. 8C). Similar experiments were performed with the PMCA1-specific antibody, which were equally unsuccessful. We also tested the possibility that a related adaptor protein, NHERF-2, might anchor PKA to the apical PMCA1. However, this was not detected using Western blotting and was thus eliminated from further investigation.

**FIGURE 9.** PKA-RIIβ translocates to the apical region of acutely isolated mouse parotid acinar cells following stimulation. Immunofluorescence experiments of methanol-fixed acutely isolated mouse parotid acinar cells showing the localization of IP3R-2 (red) and PKA-RIIβ (green) and corresponding merged images in untreated control cells (A) and following treatment with CCh (1 μM) and forskolin (Forsk) (10 μM) for 10 min (B). IP3R-2 was detected using the rabbit anti-CT-2 antibody (directed against the C-terminal domain of the IP3R-2) and the secondary AlexaFluor568-conjugated goat anti-rabbit antibody (red). PKA-RIIβ was detected using the mouse monoclonal antibody and the secondary AlexaFluor488-conjugated goat anti-mouse antibody (green). Immunofluorescent images were acquired sequentially using 488 and 568 nm laser scanning confocal microscopy. As a negative control fixed cells were incubated without any primary antibody but with the appropriate fluorescent secondary antibody alone (A, panel ii, and B, panel ii). Confocal microscope settings were the same. C, quantification of PKA-RIIβ translocation to the apical region was achieved by calculating the ratio of apical:basal fluorescence for any given cell (mean data, C, panel i) as indicated in the example (C, panel ii). Scale bars on each image represents 15 μm.
PKA-RIIβ Co-immunoprecipitated with IP₃R-2—In the absence of any direct binding of PKA-RIIβ with the PMCA, we reasoned that PKA may be targeted to the IP₃R-2, as this is regulated by PKA (6), and predominantly localized in the apical region of parotid acinar cells (17). We therefore performed similar experiments to those described above by immunoprecipitating with the RIβ antibody and Western blotting with the C2-IP₃R antibody (see Fig. 8D). Bands at the expected molecular weight for the IP₃R (~240 kDa) were clearly visible in the lysates and RIβ immunoprecipitates from untreated and treated cells. However, the band intensity was clearly greater in RIβ immunoprecipitates from cells treated with CCh and forskolin (see Fig. 8D). This suggests that the association of PKA-RIIβ with the IP₃R-2 is dynamically regulated by treatment with CCh and forskolin.

PKA-RIIβ Translocates to the Apical Region of Parotid Acinar Cells—To further investigate the nature of this dynamically regulated targeting of PKA-RIIβ to the IP₃R-2, immunofluorescence of acutely isolated cells was performed. This allowed the localization of PKA-RIIβ to be determined before and following treatment with CCh and forskolin. In untreated acutely isolated parotid acinar cells, PKA-RIIβ was distributed to all regions of the cytosol and was devoid of the nucleus as indicated by co-staining with 4′,6-diamidino-2-phenylindole (see Fig. 9A, panel i). In some cells PKA-RIIβ appeared slightly punctate and more abundant in the basal region or around the nucleus (see arrowhead, Fig. 9A, panel i). The IP₃R-2 was most abundantly localized to the apical region (red, see Fig. 9A, panel i), consistent with previous studies (17). Following treatment with CCh and forskolin, PKA-RIIβ exhibited a more complex distribution that appeared more abundant in the apical and lateral regions of the cells (see Fig. 9B, panel i). However, the distribution of the IP₃R-2 was essentially identical in both untreated and treated cells. These data suggest a dynamically regulated translocation of PKA-RIIβ to the apical region. Co-staining with both the IP₃R-2 and PKA-RIIβ showed that the co-localization or overlap was not identical, but rather PKA-RIIβ appeared to translocate to within the general vicinity of the IP₃R-2 and thus apical PMCA. This suggests that although there was an enhanced association with the IP₃R-2, not all the PKA-RIIβ associated with the IP₃R-2 but likely associated with other targets within the apical region. Attempts were made to quantify the translocation of PKA-RIIβ by measuring the relative distribution between the apical and basal parts of the same cells (see Fig. 9C, panel i). Equal sized areas of interest were selected from easily identifiable apical and non-nuclear basal regions (see Fig. 9C, panel ii). The apical regions were identified by co-staining of the IP₃R-2. Ratios of fluorescence (apical/basal) were then determined for each easily identifiable cell within each cluster per slide per experiment to yield the mean data shown in Fig. 9C, panel i. These data show that treatment with CCh and forskolin caused a 1.81 ± 0.03-fold increase (n = 11) in apical PKA relative to the corresponding basal region, compared with a 1.04 ± 0.02-fold difference in untreated control cells (*, p < 0.01, n = 11). This confirms quantitatively that indeed PKA translocates to the apical region of parotid acinar cells following treatment with CCh and forskolin.

DISCUSSION

Previous studies have shown in mouse parotid acinar cells that elevation of cAMP by forskolin and the subsequent activation of PKA potentiated [Ca²⁺]i clearance (7). Functional evidence suggested that this was due to the PMCA activity, because pharmacological inhibition of other [Ca²⁺]i clearance pathways such as SERCA and mitochondrial Ca²⁺ uptake failed to prevent the potentiation. However, under conditions where the PMCA was inhibited with La³⁺ (1 mM), the potentiation of [Ca²⁺]i clearance was abolished (7). Furthermore, biochemical evidence suggested that this potentiation was due to a Ca²⁺-dependent, PKA-mediated phosphorylation of the PMCA (7). For example, phosphorylation of the PMCA was not observed following treatment of cells with forskolin alone; however, maximum phosphorylation was observed following the combined treatment with forskolin and a [Ca²⁺]i-raising agent (CCh or CPA). Moreover, this phosphorylation was abolished by pretreatment of cells with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-tetrakis(acetoxymethyl ester). This study showed that the apical PMCA was differentially potentiated by PKA activation in both mouse parotid acinar cells and the related Par-C10 cells, which were used as a model to functionally isolate the apical and basolateral PMCA activity.

In the continued presence of forskolin, if the measurement of [Ca²⁺]i clearance was initiated at the peak of the CPA-evoked [Ca²⁺]i response, global [Ca²⁺]i clearance was largely unaffected. However, if the measurement of [Ca²⁺]i clearance was initiated at the plateau of the CPA-evoked [Ca²⁺]i response, where cells were exposed to forskolin for ~3–4 min longer, global [Ca²⁺]i clearance was potentiated. This suggests that forskolin had a time-dependent effect on [Ca²⁺]i clearance, most likely due to PMCA activity. However, closer examination of the peak response data revealed that [Ca²⁺]i clearance in the apical region was markedly potentiated and thus much faster than in the basolateral region of forskolin-treated parotid acinar cells. Furthermore, the rate of [Ca²⁺]i clearance in the basolateral region had effectively “caught up” with the apical [Ca²⁺]i clearance when clearance was initiated at the plateau of the CPA-evoked [Ca²⁺]i response. This therefore suggests that the PMCA in all regions of the plasma membrane has the capacity to be regulated by PKA activation, when cAMP levels are allowed to accumulate to high levels for prolonged periods of time. However, these data also suggest that the apical PMCA is rapidly and efficiently regulated by PKA, which may have important functional implications during dynamic signaling.

It was also noticed that the rate of increase of [Ca²⁺]i, following addition of CPA appeared slower in the apical region compared with the basal region of forskolin-treated cells. This is consistent with an almost immediate potentiation of [Ca²⁺]i clearance in the apical region, thereby slowing the rate of increase of [Ca²⁺]i, in that region. However, an alternative explanation is that store-operated Ca²⁺ influx is potentiated in the basal region. However, the time-to-peak was not significantly different in the basal regions of forskolin-treated cells compared with control cells. In addition, following removal of external Ca²⁺ at the peak response, any contribution of Ca²⁺ influx was removed, yet a faster apical clearance was clearly

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evident. Furthermore, these observations are unlikely to be due to differences in the contribution of passive ER Ca\(^{2+}\) leak between the different experimental paradigms, because the global or local [Ca\(^{2+}\)], clearances were not significantly different between the two experimental paradigms in untreated control cells. This suggests that ER Ca\(^{2+}\) leak does not significantly contribute to the measured [Ca\(^{2+}\)], clearance whatever the experimental paradigm. Nevertheless, we cannot completely rule out the possibility that forskolin treatment differentially affects ER Ca\(^{2+}\) leak in different regions of the cell, which may contribute to the observed effects.

It was therefore necessary to further test whether the apical PMCA was differentially regulated by PKA activation using a model whereby the apical and basolateral PMCA could be functionally isolated. This was achieved using Par-C10 cells grown on Transwell supports in which the apical and basolateral sides of the cell monolayer were separately perfused with La\(^{3+}\) to inhibit the PMCA. Par-C10 cells are an SV40-transformed, immortalized rat parotid acinar cell line that when grown on Transwell supports differentiate into “tight” columnar epithelial monolayers as indicated by the appearance of tripartite junctions (8). This is a very useful property as it means that the apical and basolateral sides of the cells can be separately perfused with solutions containing different agonists and ionic composition. This has been exploited and used in ion flux studies using Ussing chambers to measure short circuit currents as a model of fluid secretion (8). Par-C10 cells exhibit a predominately parotid acinar-like phenotype, including the presence of secretory granules, secretory canaliculi, expression of basolateral muscarinic and adrenergic receptors, apical purinergic receptors (P2Y\(_{j}\), CLC family of channels as well as CFTR (8). More importantly, however, this study demonstrated that forskolin markedly potentiated CCh-evoked [Ca\(^{2+}\)], signaling in Par-C10 cells, consistent with native parotid acinar cells (7). This suggests that Par-C10 cells express a similar repertoire of [Ca\(^{2+}\)], signaling machinery that makes them a useful model for making direct comparisons with native mouse parotid acinar cells. Furthermore, when grown on Transwell filters, Par-C10 cells showed clear signs of forming a tight epithelial barrier as indicated by TEER measurements of 1000–3000 ohms - cm\(^2\) similar to previous studies (8). In addition, apically applied ATP and basolaterally applied CCh evoked robust increases in [Ca\(^{2+}\)], consistent with the polarized expression of P2Y\(_{j}\) purinergic receptors and muscarinic receptors. Similar results were also obtained by measuring short circuit currents (8); however, this study is the first to demonstrate polarized agonist-evoked [Ca\(^{2+}\)], responses in Par-C10 cells.

The Par-C10 cell monolayer therefore provides a unique model for functionally isolating the apical or basolateral PMCA by applying La\(^{3+}\) to the opposite side. Using this experimental system it was found that the functionally isolated apical PMCA represented on average ∼40% of the total PMCA activity, whereas the basolateral PMCA represented ∼20% of the total PMCA activity. This therefore suggests that there is a greater PMCA activity at the apical plasma membrane consistent with previous immunofluorescence and functional studies in the related submandibular and pancreatic acinar cells (18, 19). It is difficult to reconcile the fact that the numerical sum of the functionally isolated apical and basolateral PMCA activities does not total 100% (instead ∼60%). This suggests that additional [Ca\(^{2+}\)], clearance pathways, such as mitochondrial Ca\(^{2+}\) uptake or NCX, may contribute to the measured [Ca\(^{2+}\)], clearance. Mitochondrial Ca\(^{2+}\) uptake is a low affinity, high capacity [Ca\(^{2+}\)], clearance pathway that is more likely to contribute to [Ca\(^{2+}\)], clearance during agonist-evoked Ca\(^{2+}\) release, rather than have a dominant role during a CPA-evoked [Ca\(^{2+}\)], response (20). NCX is also a low affinity, high capacity [Ca\(^{2+}\)], clearance pathway driven by the electrochemical Na\(^+\) gradient and thus has a dominant role in electrically excitable cells such as cardiac myocytes (21). To the authors’ knowledge, there is no evidence that parotid acinar cells express NCX; however, in the related pancreatic duct cells, NCX has been shown to contribute to [Ca\(^{2+}\)], clearance and to be localized to the apical membrane (22). It is therefore conceivable that a similar mechanism exists in Par-C10 cells, because of de-differentiation to a more ductal-like origin. However, in this study inhibition of mitochondrial Ca\(^{2+}\) uptake (with Ru360), and inhibition of any possible NCX activity (with NMDG), had no effect on the measured [Ca\(^{2+}\)], clearance in both parotid acinar cells and Par-C10 cells. Furthermore, the specific PMCA inhibitor, carboxyeosin (15, 16), was found to have similar effects to La\(^{3+}\), thereby further validating that the measured La\(^{3+}\)-sensitive [Ca\(^{2+}\)], clearance is due to the PMCA activity. Nevertheless, it is possible that inhibition of PMCA activity by 1 mM La\(^{3+}\) is not complete, such that any residual clearance is due to uninhibited PMCA activity. This has been shown to be the case in T cells, whereby 2 mM La\(^{3+}\) inhibited [Ca\(^{2+}\)], clearance by 90% even when all other [Ca\(^{2+}\)], clearance pathways were inhibited (12). In fact, in this study there appeared to be a small residual clearance (∼5%) even when La\(^{3+}\) was applied to both sides of the cell suggesting that this may be the case. An alternative explanation is that La\(^{3+}\) has leaked from one side of the cell layer to another because the tight junctions have become impaired such that when La\(^{3+}\) is applied to one side there is a partial inhibition of PMCA activity on the other side. However, the most striking observation from these data, which helps to reconcile these discrepancies, is that forskolin massively increased the functionally isolated apical PMCA activity, whereas the basolateral PMCA activity was unaffected. If La\(^{3+}\) inhibited the apical PMCA when applied to the basolateral side of the cells, and vice versa, then such large differences in forskolin effectiveness would not have been observed. Likewise, an effect of forskolin on mitochondrial [Ca\(^{2+}\)], uptake is unlikely to account for these results as one would expect this to be the same no matter which side La\(^{3+}\) was applied. In addition, forskolin failed to affect [Ca\(^{2+}\)], clearance when the PMCA was inhibited by La\(^{3+}\) in mouse parotid acinar cells (7). Collectively, these data provide convincing functional evidence that the apical PMCA is differentially potentiated by PKA activation in Par-C10 cells, consistent with our observations in mouse parotid acinar cells.

The PMCA is ubiquitously expressed and has a high affinity for Ca\(^{2+}\) suggesting that it has a major role in the maintenance of resting [Ca\(^{2+}\)], (23, 24). In addition, evidence suggests that the PMCA is regulated by dynamic changes in [Ca\(^{2+}\)], during normal [Ca\(^{2+}\)], signaling (12, 25). PKA-mediated phosphorylation of PMCA has been shown previously to increase the
PMCA activity by increasing the affinity for calmodulin binding (26). Furthermore, in parotid acinar cells, activation of PKA potentiates PMCA activity in a Ca<sup>2+</sup>-dependent manner, thereby tuning its activity so that it becomes exquisitely sensitive to [Ca<sup>2+</sup>] (7). Together, these data indicate that PKA-mediated phosphorylation of the PMCA may be an important mechanism by which cAMP shapes the temporal properties of [Ca<sup>2+</sup>], signaling. This present study also suggests that such Ca<sup>2+</sup>-dependent, PKA-mediated regulation of the apical PMCA may give the cell exquisite control over the spatial properties of [Ca<sup>2+</sup>], signaling.

Four PMCA genes have been identified and cloned; PMCA1 and -4 are ubiquitously expressed, whereas PMCA2 and -3 are expressed predominantly in excitable cells (27). There are also over 20 splice variants some of which have differential PKA and calmodulin sensitivity (28). In this study, PMCA1, -2, -4 were shown to be expressed in parotid acinar cells using Western blotting techniques. Moreover, immunofluorescence experiments revealed that PMCA1 was localized to all regions of the plasma membrane; PMCA2 was confined to the basolateral membrane, and PMCA4 was confined to the apical membrane. However, in situ phosphorylation experiments revealed that the only PMCA isoform to be phosphorylated following the combined treatment of forskolin and CCh was PMCA1. This is consistent with biochemical studies that have shown that the splice variant PMCA1b is the only isoform that contains a good PKA consensus site (KRNSS) and can be phosphorylated by PKA (29, 30). PMCA2b contains a relatively poor PKA consensus site (KQNSS) and is only weakly phosphorylated, whereas the corresponding region in PMCA4b (KASKF) is not known to be regulated by PKA (29, 30). In addition, the "a" splice variants of all the PMCA isoforms have this region spliced out and are therefore not thought to be "conventionally" regulated by PKA (31).

This suggests that PMCA1 is the most likely candidate responsible for the differential regulation of the apical PMCA by PKA. However, this presents a functional paradox. How does PKA differentially regulate PMCA1 at the apical membrane when PMCA1 is distributed to all regions of the plasma membrane? One possibility is that PKA is targeted to PMCA1 at the apical membrane, via a scaffolding or anchoring protein, thus facilitating the efficient regulation of the pump. The most likely candidates for such regulation are the protein kinase A anchoring proteins (AKAPs), described for the targeted PKA regulation of numerous substrate proteins (32). There are numerous AKAPs that can target PKA to different regions of the cell allowing the efficient regulation of specific substrates (32). AKAP79/150 has been shown to target PKA to the plasma membrane, via a phosphatidylinositol 4,5-bisphosphate binding domain, and thus represents a potential candidate to target PKA to the PMCA. Although AKAP79/150 is expressed in parotid acinar cells, it has been shown to be localized to the basolateral membrane, not the apical membrane, where it associates with the Na<sup>+</sup>/K<sup>+</sup>-ATPase (33). Other potential candidate proteins that may target PKA to the apical PMCA1 are ezrin and/or EBP50 because immunofluorescence experiments showed that both ezrin and EBP50 were localized to the apical region of mouse parotid acinar cells. Ezrin is a member of the ERM (Ezrin/Radixin/Moesin) family of proteins that couple the cortical actin cytoskeleton (F-actin) to plasma membrane-bound proteins via the PDZ domain proteins EBP50 (ezrin-binding protein of 50 kDa) or NHERF-2 (Na<sup>+</sup>/H<sup>+</sup> exchange regulatory factors-2) (34–36). In epithelial cells, ezrin is important for the formation of apical microvillae and in conferring cellular polarity (36). EBP50 is an adaptor protein that binds ezrin and behaves as a protein kinase A-anchoring protein important for the targeted PKA-mediated regulation of a variety of important integral plasma membrane proteins, such as the CFTR (37), Na<sup>+</sup>/H<sup>+</sup> exchanger (38), and platelet-derived growth factor receptors (39). The PMCA contains a PDZ (PSD-95/Dlg/ZO-1) binding domain that binds to multiprotein signaling complexes (40–44) important for maintaining micro-domains of [Ca<sup>2+</sup>], and thus local regulation of Ca<sup>2+</sup>-dependent effectors (40, 41, 45). Therefore, it was conceivable that PMCA1 may be regulated by PKA at the apical membrane by an indirect PDZ-mediated interaction with ezrin and/or EBP50/NHERF-2. However, this study found that neither PKA-RIIβ itself, ezrin, EBP50, nor NHERF-2 could co-immunoprecipitate with the PMCA, either at rest or following stimulation with CCh and forskolin. These data therefore suggest that the differential regulation of the apical PMCA does not involve a direct association of PKA with the apical PMCA via any anchoring protein.

Another possible explanation is that PKA is targeted in close proximity to the apical PMCA, perhaps by a direct association with another substrate that resides predominantly in the apical region. Although there are likely numerous potential substrates for PKA in the apical region of parotid acinar cells, we reasoned that the IP<sub>3</sub>R-2 may be a potential candidate. This is because Ca<sup>2+</sup> release from the IP<sub>3</sub>R-2 is markedly potentiated by PKA-mediated phosphorylation (7) and is abundantly localized to the apical region of parotid acinar cells (17). Indeed, in this study, PKA-RIIβ co-immunoprecipitated with the IP<sub>3</sub>R-2, which was markedly enhanced following treatment of cells with CCh and forskolin. This is an important mechanistic observation as it suggests that PKA is not simply anchored to its substrate as a fixed static signaling complex, but rather the assembly of such a complex may be dynamically regulated by other signaling pathways, such as Ca<sup>2+</sup> or even cAMP/PKA itself. Moreover, immunofluorescence experiments demonstrated that treatment with CCh and forskolin also caused PKA-RIIβ to translocate to the apical region of parotid acinar cells. This further supports the notion that PKA is not a static entity and that both its activity and localization can be dynamically regulated. This regulated translocation of PKA did not completely co-localize with the IP<sub>3</sub>R-2, but rather it translocated to within the general vicinity of the apical and lateral regions of the cells. This suggests that PKA may dynamically associate with other PKA-dependent effectors within the apical region and does not exclusively associate with IP<sub>3</sub>R-2. These include, for example, the CFTR (46, 47) or the exocytotic machinery (48), which is under synergistic control by both [Ca<sup>2+</sup>], and cAMP (48). In addition, recent studies have demonstrated that PKA-RIIα associates with aquaporin (AQP2)-containing vesicles, via a splice variant of AKAP18 (AKAP188) (49). This facilitates the translocation and subsequent fusion of AQP2 with the apical
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membrane of kidney collecting duct cells, the major mechanism of water reabsorption in the kidney (49). A similar mechanism involving the translocation of AQP5 to the apical membrane is believed to be important in the control of fluid secretion in the parotid gland (48). However, it remains controversial as to whether this is primarily controlled by Ca$^{2+}$ or cAMP (48). Translocation of PKA via the dynamic regulation of specific anchoring proteins is not an unprecedented phenomenon. For example, RII and actin compete for overlapping regions on the anchoring protein, Wiskott-Aldrich verpolin-homology protein-1 (WAVE1), suggesting that under certain situations PKA anchoring to WAVE1 may be favored over tethering to the actin cytoskeleton (50). Moreover, the AKAP18α and -β have been shown to specifically associate with either the lateral or apical membrane of Madin-Darby canine kidney cells, via specific myristoylation or palmitoylation, which in turn are dynamic and reversible processes (51). Furthermore, protein kinase C-mediated phosphorylation of AKAP79/150 has been shown to cause dissociation from the membrane, suggesting that PKA may translocate to and from the membrane under different situations (52). Whatever the molecular mechanism, these observations add another layer of complexity to the fidelity of signaling and signaling cross-talk and may represent a general mechanism for the regulation of numerous Ca$^{2+}$- and cAMP/PKA-dependent effectors in a variety of cells. In addition, and of relevance to this study, these phenomena help to provide further molecular evidence not only for the potentiation of Ca$^{2+}$ release (6), but also for the differential regulation of the apical PMCA in parotid acinar cells.

This study therefore suggests that in addition to shaping the temporal properties of [Ca$^{2+}$], signals, PKA-mediated regulation of the PMCA in parotid acinar cells may also contribute to the spatial shaping of the [Ca$^{2+}$], signals. The PMCA has been shown to be predominantly apically located in the structurally and functionally related pancreatic and submandibular acinar cells (18) and is more pronounced across the apical membrane of pancreatic acinar cells (19). This suggests that the apical PMCA is functionally more important in these related cells, because many Ca$^{2+}$-dependent effectors reside within or are very close to the apical membrane. These include the exocytotic machinery and the Ca$^{2+}$-dependent Cl$^-$ channels, which are arguably the most important functional Ca$^{2+}$-dependent effectors in these secretory cells (6, 53). Therefore, PKA-mediated regulation of the apical PMCA may give the cell further control over these important Ca$^{2+}$-dependent effectors. This may be particularly important within the highly invaginated microvillar structures of the apical membrane, whereby the PMCA may be the only means for the clearance of local concentrations of Ca$^{2+}$ because of the absence of ER (37).

In parotid acinar cells (54) and other exocrine acinar cells (18, 55), the apical region is regarded as the “trigger zone” from which Ca$^{2+}$ waves are initiated. This is partly due to a highly enriched localization of IP$_3$R at the extreme apical region (see Fig. 9) (17, 56). Furthermore, PKA-mediated phosphorylation of IP$_3$R-2, which is also enriched at the apical pole (17), leads to the marked potentiation of Ca$^{2+}$ release in parotid acinar cells (6). It may seem counterintuitive to potentiate [Ca$^{2+}$], clear-

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