Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors in Parotid Acinar Cells

A MECHANISM FOR THE SYNERGISTIC EFFECTS OF cAMP ON Ca2+ SIGNALING*

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Acetylcholine-evoked secretion from the parotid gland is substantially potentiated by cAMP-raising agonists. A potential locus for the action of cAMP is the intracellular signaling pathway resulting in elevated cytosolic calcium levels ([Ca2+]i). This hypothesis was tested in mouse parotid acinar cells. Forskolin dramatically potentiated the carbachol-evoked increase in [Ca2+]i, converted oscillatory [Ca2+]i changes into a sustained [Ca2+]i increase, and caused subthreshold concentrations of carbachol to increase [Ca2+]i measurably. This potentiation was found to be independent of Ca2+ entry and inositol 1,4,5-trisphosphate (InsP3) production, suggesting that cAMP-mediated effects on Ca2+ release was the major underlying mechanism. Consistent with this hypothesis, dibutyryl cAMP dramatically potentiated InsP3-evoked Ca2+ release from streptolysin-O-permeabilized cells. Furthermore, type II InsP3 receptors (InsP3R) were shown to be directly phosphorylated by a protein kinase A (PKA)-mediated mechanism after treatment with forskolin. In contrast, no evidence was obtained to support direct PKA-mediated activation of ryanodine receptors (RyRs). However, inhibition of RyRs in intact cells, demonstrated a role for RyRs in propagating Ca2+ oscillations and amplifying potentiated Ca2+ release from InsP3Rs. These data indicate that potentiation of Ca2+ release is primarily the result of PKA-mediated phosphorylation of InsP3Rs, and may largely explain the synergistic relationship between cAMP-raising agonists and acetylcholine-evoked secretion in the parotid. In addition, this report supports the emerging consensus that phosphorylation at the level of the Ca2+ release machinery is a broadly important mechanism by which cells can regulate Ca2+-mediated processes.

Calcium is a ubiquitous second messenger that is critically important in the regulation of a variety of cellular functions (1–3). The spatio-temporal “shaping” of Ca2+ signals is thought to play an important role in defining the specificity of stimulus-response coupling both between cell types and within the same cell (4, 5). However, despite intensive investigation, the molecular mechanisms that control frequency- and/or amplitude-encoded Ca2+ oscillations, Ca2+ wave propagation, or localized Ca2+ release events remain poorly understood. An emerging body of evidence indicates that, in various systems, specific control over Ca2+ signals may be achieved by cross-talk between second messenger systems that raise [Ca2+]i, interacting with those that elevate cAMP. Such cross-talk may alter the sensitivity of a variety of Ca2+ transport processes (6, 7).

An example of this cross-talk occurs in the salivary gland, where both fluid and exocytotic secretion are controlled by separate neuronal and/or hormonal inputs (6, 8). Specifically, neuronally released acetylcholine (ACh) activates acinar cell muscarinic receptors, leading to increased [Ca2+]i, via the phosphoinositide pathway. Elevations in [Ca2+]i, activate ion channels essential for unidirectional fluid secretion (9), and, in addition, exert regulatory control over the exocytotic machinery required for protein secretion (6). Muscarinic activation of both fluid secretion, and to a lesser extent exocytosis, has been shown to be dramatically potentiated by the concomitant activation of cAMP-raising pathways, such as by co-released vasoactive intestinal peptide, or by sympathetic stimulation of β-adrenoreceptors (10–13). Although cAMP could have direct effects on ion channels (14) and/or exocytotic proteins (15), an alternative hypothesis is that cAMP interacts directly with the Ca2+ signaling machinery to account for the synergistic effects (6, 13, 16). Because parotid acinar cells have been used extensively to study Ca2+ signaling, this model system is ideally suited to investigate cross-talk between cAMP and Ca2+ signaling. InsP3 production, Ca2+ influx, and Ca2+ release from either InsP3Rs or RyRs, are all potential targets for cAMP in modulating Ca2+ signaling, however, the literature is equivocal as to the site of any interaction (16–23). No single study has been successful in unambiguously identifying a specific molecular target that accounts for the synergistic relationship between cAMP and Ca2+ signaling in parotid acinar cells. Therefore, the aim of the present study was to investigate the molecular target(s) for the interaction between cAMP and Ca2+ signaling in mouse parotid acinar cells. This was achieved using a combination of imaging (intact and SL-O-permeabilized cells), inositol phosphate assays and in situ phosphorylation experi-

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The abbreviations used are: [Ca2+]i, intracellular calcium concentration; InsP3, inositol 1,4,5-trisphosphate; InsP7R, inositol 1,4,5-trisphosphate receptor; ACh, acetylcholine; CCH, carbamylcholine (carbachol); PKA, protein kinase A; RyR, ryanodine receptor; Bt2cAMP, dibutyryl cAMP; SL-O, streptolysin-O, CICR, calcium-induced calcium release; PLC, phospholipase C; Cmc4, 4-cholamidopropyldimethylammonium)-1-propanesulfonic acid; RP-cAMPS, Rp-adenosine-3′,5′-cyclic monophosphorothioate.

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ments. These experimental paradigms revealed that cAMP dramatically potentiated Ca\textsuperscript{2+} release through PKA-mediated phosphorylation of InsP\textsubscript{3} receptors, likely the type II isoform. This regulatory control likely underlies the synergistic relationship between ACh and cAMP-elevating agonists in parotid acinar cells. These findings have broad implications and may represent a general feature for the regulation of Ca\textsuperscript{2+} release events that are linked to a vast array of specific functions in all cell types.

**EXPERIMENTAL PROCEDURES**

**Isolation of Single Parotid Acinar Cells**—Single and small groups of parotid acinar cells were isolated by collagenase digestion of freshly dissected parotid glands from wild type Swiss Black mice using a technique similar to that described previously for rat parotid (24). Briefly, 25-g mice were killed by cardiac puncture immediately following CO\textsubscript{2} asphyxiation. Parotid glands were dissected, minced, and incubated for 60 min at 37 °C in Earle’s minimum essential medium (Biofluids, Inc., Rockville, MD) containing 2 mM glutamine, 1% bovine serum albumin, and 0.04 mg/ml collagenase P (Roche Molecular Biochemicals, Mannheim, Germany). Minced tissue was dispersed by multiple trituration every 20 min. Cells were resuspended in bovine serum albumin-free Eagle’s basal medium (Invitrogen) supplemented with 2 mM glutamine and penicillin/streptomycin and left on ice until ready for use.

**Digital Imaging of [Ca\textsuperscript{2+}]\textsubscript{i}**.—Isolated parotid acinar cells were resuspended in a HEPES-buffered physiological saline solution (HEPES-PSS) containing (in mM) 5.5 glucose, 137 NaCl, 0.56 MgCl\textsubscript{2}, 4.7 KCl, 1 Na\textsubscript{2}HPO\textsubscript{4}, 10 HEPES (pH 7.4), 1.2 CaCl\textsubscript{2}. The cells were then incubated in the above HEPES-PSS containing 2 μM fura-2/AM for 30 min at room temperature, after which they were washed once and resuspended in the above HEPES-PSS and kept on ice. Cells were allowed to adhere to a glass coverslip that formed the base of a gravity-fed perfusion chamber and continually perfused with HEPES-PSS. [Ca\textsuperscript{2+}]\textsubscript{i} imaging was performed using an inverted epifluorescence Nikon microscope with a 40×/1.3 oil immersion objective lens (26). Images were acquired every second with an exposure of 200 ms. Background-subtracted and ratio images were calculated on-line and stored for Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} release experiments were similar to those reported in pancreatic acinar cells (26, 27).

**Measurement of Intracellular [Ca\textsuperscript{2+}]**.—Isolated parotid acinar cells were incubated in the above attachment media containing 10 μM EGTA, 0.5 mM nitriloacetic acid, and 20 mM HEPES/KOH (pH 7.1). Following incubation, cells were washed three times in the above HEPES-PSS-free media and aliquots treated with or without 10 μM forskolin for 30 min at 37 °C. Cells were rapidly pelleted by centrifugation and resuspended in ice-cold lysis buffer containing (in mM) 50 Tris-HCl (pH 7.4), 250 NaCl, 5 EDTA, 100 NaF, 1 benzamidine, 1 dithiothreitol, 1 mM CHAPS, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 1 μg/ml aproxin, 1 μg/ml antipain. Cell lysates were then sonicated, left on ice for 30 min, and vortexed every 5 min. InsP\textsubscript{R} or RyR protein was immunoprecipitated from the samples by incubating lysates with a mixture of antibodies (1 μg of antibody/mg of protein) raised against all three InsP\textsubscript{R} types or all three RyR types for 1 h at 4 °C, followed by incubation with 80 μl of protein A-agarose beads (Pierce) for an additional 1 h at 4 °C. Antibodies directed against InsP\textsubscript{R} were CT\textsubscript{1} and CT\textsubscript{5} (kind gifts from Richard Wojcikiewicz, State University of New York, Syracuse, NY) and type III antibody (Transduction Laboratories). Antibodies directed against RyR were 34C directed against RyR\textsubscript{1} (Devol- opmental Studies Hybridoma Bank, Iowa City, IA), C3–33 directed against RyR\textsubscript{2} (Affinity Bioreagents, Inc.), and anti-rabbit skeletal muscle RyR antibody directed against RyR\textsubscript{3} (Upstate Biotechnology, Lake Placid, NY). As a secondary control, aliquots of cell lysate from cells treated with forskolin were incubated with beads without any antibody.

Phospho-PKA substrate antibodies were used to implicate a specific role of PKA. Phosphoproteins containing phosphorylated serine or threonine, with an arginine residue at position +3, but not the corresponding nonphosphorylated motif (29, 30). These antibodies were used in combination with an additional and complimentary approach using a phospho-PKA substrate antibody (Cell Signaling Technology). This antibody specifically recognized phosphoproteins containing phosphorylated serine or threonine, with an arginine residue at position +3, but not the corresponding nonphosphorylated motif (29, 30). This antibody would not discriminate between substrates of PKA, protein kinase C, or cyclic GMP-dependent protein kinase, the combined use of forskolin and appropriate PKA inhibitors was used to implicate a specific role of PKA.

Aliquots of isolated parotid acinar cells were treated with or without 10 μM forskolin, 50 mM okadaic acid, and/or the PKA inhibitors, H-89 (2 μM), Rp-cAMPs (30 μM) for 10 min at 37 °C. Cells were then solubilized in lysis buffer similar to the method above. Lysates were then incubated with phospho-PKA substrate antibody (1:100 dilution) to immunoprecipitate phosphorylated proteins. Specific detection of phosphorylated type II InsP\textsubscript{R} was achieved by Western blotting with the CT\textsubscript{1} antibody. Whole cell lysates or immunoprecipitated proteins were denatured in SDS-sample buffer containing (in mM) 62.5 Tris-HCl, 2% SDS, 10% glycerol, 5% [32P]orthophosphate and 1% β-mercaptoethanol then boiled for 5 min followed by centrifugation to remove insoluble material. Aliquots were loaded on SDS-PAGE and exposed to a PhosphorImager intensifier screen. Bands of the appropriate molecular weight, which corresponded to phosphorylated proteins, were cut out and separated, dried, and subjected to liquid scintillation counting.

**Data Analysis and Experimental Design**—In all experiments (unless

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**Cyclic AMP and [Ca\textsuperscript{2+}]\textsubscript{i} Signaling**
RESULTS

Carbachol-evoked \([\text{Ca}^{2+}]_i\), Changes—To investigate the effects of elevated cAMP levels on \([\text{Ca}^{2+}]_i\), signaling in parotid acinar cells, we first characterized the types of \([\text{Ca}^{2+}]_i\), responses evoked by the muscarinic receptor agonist, CCh. Low concentrations of CCh (10–300 nM) caused an oscillatory increase in \([\text{Ca}^{2+}]_i\), in 86% of cells tested, which was characterized by a large initial spike followed by rapid sinusoidal oscillations superimposed over an elevated base line. These oscillations were complex in nature, and their frequency and amplitude varied markedly between cells. Higher concentrations of CCh (300 nM to 10 \(\mu\)M) induced a biphasic increase in \([\text{Ca}^{2+}]_i\), which was characterized by a large initial spike followed by a sustained elevation. These patterns of \([\text{Ca}^{2+}]_i\), changes are typical of a variety of exocrine cells; however, the oscillation frequency was significantly higher than reported in pancreatic acinar cells (7–11/min in parotid compared with 4–6/min; see Ref. 31).

Despite the complex nature of these CCh-evoked \([\text{Ca}^{2+}]_i\), changes, there was a consistent concentration-dependent increase in the magnitude of the initial spike-like increase in \([\text{Ca}^{2+}]_i\). This initial \([\text{Ca}^{2+}]_i\), spike was interpreted to reflect \(\text{Ca}^{2+}\) release from intracellular stores and was quantitatively compared in the absence and presence of 10 \(\mu\)M forskolin using a paired experimental design.

Forskolin Potentiates Carbachol-evoked \([\text{Ca}^{2+}]_i\), Changes—Using the adenylyl cyclase activator forskolin, we investigated the effects of elevating intracellular cAMP levels on CCh-evoked \([\text{Ca}^{2+}]_i\), signaling (33). Repeated stimulations with CCh evoked \([\text{Ca}^{2+}]_i\), changes of equal magnitude (Fig. 1). Forskolin (10 \(\mu\)M) induced a dramatic and time-dependent potentiation of this CCh-evoked initial increase in \([\text{Ca}^{2+}]_i\). Upon removal of forskolin, there was an equivalent time-dependent recovery (Fig. 1). On average, forskolin increased the CCh-evoked \([\text{Ca}^{2+}]_i\), response by 148.9 \pm 8.5% after 3–5 min, which increased further to 177.1 \pm 17.4% after 8–10 min of incubation with forskolin (see Fig. 1). To test whether the potentiation was the result of specific activation of PKA, cells were also pretreated with 2 \(\mu\)M H-89, an inhibitor of serine/threonine kinases such as PKA (34) prior to specific activation of PKA by treatment with forskolin. This completely prevented the potentiation of the CCh-evoked initial increase in \([\text{Ca}^{2+}]_i\), (Fig. 2), suggesting that the potentiation was caused by activation of PKA.

In addition to the potentiation of the CCh-evoked initial increase in \([\text{Ca}^{2+}]_i\), forskolin also converted oscillatory \([\text{Ca}^{2+}]_i\), changes into a sustained increase, suggesting a leftward shift in the CCh concentration-response curve compared with control cells (Fig. 3A). Consistent with this hypothesis, forskolin treatment enabled normally subthreshold concentrations of CCh (3–30 nM) to evoke oscillatory \([\text{Ca}^{2+}]_i\), responses (49 of 75 cells). Interestingly, in six of these cells, \([\text{Ca}^{2+}]_i\), oscillations were confined to the apical region of the cells (Fig. 3B), revealing conversion of a subthreshold response into a measurable threshold response. To identify the molecular site at which this potentiation was manifested, we systematically investigated the effects of forskolin on \(\text{Ca}^{2+}\) entry, InsP\(_3\) production, and \(\text{Ca}^{2+}\) release channels in parotid acinar cells using a variety of biochemical and functional assays.
enhancement of InsP₃ generation. To test this idea we examined the effects of forskolin on inositol phosphate production by measuring [³H]inositol incorporation to assess PLC activity and thus InsP₃ production. CCh (1 μM) significantly increased inositol phosphates from 5.9 ± 0.2 to 9.8 ± 0.1% of total phosphoinositides (Fig. 5). In contrast, forskolin (10 μM) had no effect on either basal (6.1 ± 0.1%) or CCh-evoked (8.9 ± 0.3%) inositol phosphate turnover (Fig. 5). This indicated that increased cAMP does not directly stimulate the production of inositol phosphates and suggests a likely site of action is the Ca²⁺ release process itself.

Effects of Ryanodine on Carbachol-evoked [Ca²⁺], Changes—In nonexcitable cells, InsP₃Rs are generally thought to be the trigger for Ca²⁺ release, whereas RyRs may have a role in propagating further release by Ca²⁺-induced Ca²⁺ release (CICR) (35). We pharmacologically separated these two Ca²⁺ release pathways by inhibiting RyRs with 500 μM ryanodine (36). Ryanodine alone failed to significantly affect the initial increase in [Ca²⁺], evoked by CCh (101.5 ± 7.3%; Fig. 6, A and B), suggesting that this event does not involve RyRs. However, when applied during a train of Ca²⁺ oscillations, ryanodine dramatically dampened the oscillatory [Ca²⁺], response (Fig. 6, A and B). These data therefore imply that RyRs may be important for propagating and maintaining Ca²⁺ oscillations in parotid acinar cells as is the case in pancreatic acinar cells (32).

When applied in combination with 10 μM forskolin, ryanodine significantly reduced the potentiation of the CCh-evoked [Ca²⁺], response from 177.1 ± 17.4% to 125.2 ± 2.8% of that observed in the absence of forskolin and ryanodine (Fig. 6, B and C). Nevertheless, the residual potentiation (125.2 ± 2.8%) remained significantly different from control. One possible interpretation of these data is that the potentiation of Ca²⁺ release by forskolin is caused by a direct effect of PKA on both RyRs and InsP₃Rs.

**Figure 2.** A PKA inhibitor prevents the potentiation of the CCh-evoked [Ca²⁺], response by forskolin. Using a similar experimental paradigm as Fig. 1, cells were pretreated with 2 μM H-89 for 5 min prior to treatment with 10 μM forskolin in combination with 2 μM H-89 for at least 5 min. A, representative trace from 6 separate experiments (34 cells). B, quantification of mean data revealed that the PKA inhibitor, H-89 completely prevented the potentiation by forskolin. Statistical significance was determined using a paired one sample t test (*p < 0.05).
Fig. 4. **Ca\(^{2+}\) entry is not directly affected by forskolin treatment.** A, representative experiment showing the effect of removal and subsequent re-introduction of external [Ca\(^{2+}\)] on the CCh-evoked [Ca\(^{2+}\)] response in the absence and presence of forskolin. The dotted line represents a gap of 7 to 9 min during which no images were acquired. B, quantification of mean data revealed that the initial CCh-evoked [Ca\(^{2+}\)], increase was dramatically potentiated by forskolin even in the absence of external [Ca\(^{2+}\)] (171 ± 13% increase, 4 experiments, 14 cells; *p < 0.05 determined by paired one sample t test). However this was not significantly different from the potentiation of the initial CCh-evoked [Ca\(^{2+}\)] increase by forskolin in the presence of external [Ca\(^{2+}\)] (177 ± 17% increase from Fig. 1, statistical significance determined by unpaired Mann Whitney test).

Fig. 5. **InsP\(_3\) generation is not directly affected by forskolin treatment.** [\(^{3}H\)]Insitol phosphate production, measured by incorporation of [\(^{3}H\)]Insitol into phospholipid, was assessed as an indirect measure of InsP\(_3\) generation. Inositol phosphate production was expressed as % of total phosphoinositides. 1 μM CCh significantly increased inositol phosphates from 5.9 ± 0.2% to 9.8 ± 0.1% of total phosphoinositides. Forskolin alone (6.1 ± 0.1%) or in combination with 1 μM CCh (8.9 ± 0.3%) did not significantly affect inositol phosphate production. Statistical significance was determined by unpaired t test (*p < 0.05, n = 3).

indicates that RyR-mediated Ca\(^{2+}\) release in mouse parotid acinar cells is not directly affected by raising cAMP with forskolin, and suggests that PKA has a direct effect on InsP\(_3\)Rs. The role of RyRs may simply be to amplify the enhanced Ca\(^{2+}\) release from InsP\(_3\)Rs by CICR.

**InsP\(_3\)-evoked Ca\(^{2+}\) Release from SL-O-permeabilized Cells**—To directly assess the effects of cAMP on InsP\(_3\)-evoked Ca\(^{2+}\) release, SL-O-permeabilized cells were used. Despite permeabilization, cells retained their polarity as indicated by apically located secretory granules. Following permeabilization, fluorescence became both dramatically reduced (<20%) and highly punctate, indicative of dye trapped within organelles (26, 42). Perfusion of permeabilized cells with a “cytosolic-like” medium devoid of Ca\(^{2+}\) and ATP caused a slow decline in the fura-2PF-340/380 ratio, which stabilized in 5–10 min, indicating Ca\(^{2+}\) store depletion. The subsequent addition of 0.2 μM Ca\(^{2+}\) and 1 mM Mg-ATP evoked a rapid increase in fura-2PF-340/380 ratio that reached a steady state within 3 min (Fig. 8, A–E), reflecting rapid uptake of Ca\(^{2+}\) into the ER. The observed rate was significantly faster than that reported in pancreatic acinar cells (~10 min to reach steady state; see Refs. 26 and 27).

Following Ca\(^{2+}\) uptake, InsP\(_3\) (0.1–10 μM) evoked a concentration-dependent stepwise decrease in [Ca\(^{2+}\)]

Fig. 6. **Effects of inhibition of RyRs on CCh-evoked [Ca\(^{2+}\)] responses and the potentiation by forskolin.** A, 500 μM ryanodine dampened the oscillatory [Ca\(^{2+}\)] response but failed to significantly affect the initial CCh-evoked [Ca\(^{2+}\)] increase. B, using a similar experimental paradigm and in combination with 10 μM forskolin, ryanodine reduced the potentiation of the initial CCh-evoked [Ca\(^{2+}\)], increase by forskolin. The dotted line represents a gap of 7 to 9 min during which no images were acquired. C, quantification of mean data confirmed that ryanodine failed to significantly affect the initial CCh-evoked [Ca\(^{2+}\)] increase (101.5 ± 7.3% increase), but significantly reduced the potentiation by forskolin (125.2 ± 2.8% increase compared with 177.1 ± 17.4% in the absence of ryanodine; p < 0.05 as determined by Mann Whitney test). However the residual potentiation in the presence of both ryanodine and forskolin remained significant (p < 0.05 as determined by one sampled t test).
pre-stimulatory levels, suggesting Ca\(^{2+}\) was rapidly taken back up into the ER (Fig. 8, A–F). This allowed the application of a general experimental paradigm consisting of stimulating with a low dose of InsP\(_3\) (0.3 \(\mu\)M), which evoked 24.2 \(\pm\) 0.1% maximal release, followed by a high dose (3 \(\mu\)M), which evoked nearly maximal release (93.1 \(\pm\) 0.2%; Fig. 8F, inset). This paradigm was then repeated in the presence of 100 \(\mu\)M Bt2cAMP (Fig. 8B) with or without 10 \(\mu\)M Rp-cAMPS or 500 \(\mu\)M ryanodine (Fig. 8, C–E). Time-matched controls were performed to account for loss of dye, photobleaching, or possible desensitization (Fig. 8A). Fig. 8F shows the mean data expressed as -fold increase in resting [Ca\(^{2+}\)] evoked by CmC; (three experiments, 14 cells). F, 10 \(\mu\)M forskolin failed to affect 300 \(\mu\)M CmC-evoked increase in resting [Ca\(^{2+}\)], (5 experiments, 33 cells).

**In Situ Phosphorylation of InsP\(_3\)R and RyRs—**To examine whether the potentiation of Ca\(^{2+}\) release correlated with PKA-mediated phosphorylation of InsP\(_3\)Rs and/or RyRs, two complimentary approaches were adopted using receptor specific antibodies to immunoprecipitate potentially phosphorylated protein. First, cells were metabolically labeled with \(^{32}\)PO\(_4\) and protein that had incorporated \(^{32}\)PO\(_4\) upon phosphorylation was detected by autoradiography. Following treatment with forskolin and subsequent immunoprecipitation with InsP\(_3\)R-specific antibodies, there was enhanced labeling of bands at the expected molecular weight for InsP\(_3\)Rs compared with control (Fig. 9A). This demonstrates that InsP\(_3\)Rs are directly phosphorylated, presumably by a PKA-mediated process. In contrast, under similar conditions using RyR-specific antibodies, no detectable signal was observed at the appropriate molecular weight for RyRs. Immunoprecipitation of RyR protein from skeletal muscle, cardiac muscle, and brain tissue lysates confirmed the avidity of RyR-specific antibody (data not shown).

Second, an alternative and complimentary approach used the phospho-PKA substrate antibody to immunoprecipitate phosphorylated protein, followed by specific detection of phosphorylated type II InsP\(_3\)R by immunoblot with the CT\(_2\) antibody. The specificity of the phospho-PKA substrate antibody for phosphorylated protein was verified in Fig. 9B. A variety of proteins of different molecular weight were detected upon treatment with forskolin compared with untreated cells even though equal amounts of total protein were added into each lane (Fig. 9B, lane 3 versus lane 1). The phospho-PKA substrate antibody does not distinguish between substrates of PKA, protein kinase C, and cyclic GMP-dependent protein kinase (29, 30). To distinguish between activation of these kinases, cells were treated with the PKA inhibitors H-89 (2 \(\mu\)M) and Rp-cAMPS (30 \(\mu\)M) prior to and in the continued presence of forskolin to specifically activate PKA. This completely prevented the detection of visible bands (Fig. 9B, lane 4), which strongly suggests that forskolin evokes PKA-mediated phosphorylation of proteins. Furthermore, treatment of cells with the protein phosphatase inhibitor okadaic acid (50 nM) did not dramatically increase protein phosphorylation, suggesting that basal protein phosphatase activity was low and not affected by cell lysis (Fig. 9B, lanes 5 and 6). Finally, immunoprecipitation with the phospho-PKA substrate antibody and subsequent immunoblot with
CT2 antibody showed that forskolin dramatically increased the detection of phosphorylated type II InsP$_3$R (Fig. 9C, lanes 4 and 5), which was prevented by pretreatment with H-89 and Rp-cAMPS (Fig. 9C, lanes 6 and 7). Taken together, these data provide convincing evidence that forskolin causes PKA-mediated phosphorylation of type II InsP$_3$R.

**DISCUSSION**

The present study provides functional and molecular evidence defining a specific site at which cAMP-raising agonists exert synergistic regulatory control over fluid secretion and exocytosis in mouse parotid acinar cells stimulated by muscarinic activation of [Ca$^{2+}$], signaling (10–13, 16). The signal transduction pathway following muscarinic receptor stimulation and leading to an increase in intracellular Ca$^{2+}$ is a rich source of sites for possible PKA regulation, which could account for the dramatic PKA-mediated potentiation of the CCh-evoked initial [Ca$^{2+}$], increase. The effects of raising cAMP on various aspects of this signaling pathway were therefore systematically investigated. Specifically, Ca$^{2+}$ entry has been suggested to be important not only in replenishing the stores with Ca$^{2+}$ but also in maintaining agonist-evoked sustained [Ca$^{2+}$], signals (4, 46), and in modulating the frequency of [Ca$^{2+}$], oscillations by sensitizing Ca$^{2+}$ release (47). In addition, in hepatocytes (48) and submandibular acinar cells (49), cAMP has been shown to potentiate InsP$_3$ production. However, in the present study, forskolin treatment was shown to have no appreciable effect on Ca$^{2+}$ entry or CCh-evoked PLC activity and InsP$_3$ production. These data led us to conclude that the primary site of action of PKA is on the Ca$^{2+}$ release process itself.

InsP$_3$-evoked Ca$^{2+}$ release from InsP$_3$Rs underlies agonist-induced increases in [Ca$^{2+}$], in a variety of nonexcitable cells including secretory epithelia (4, 32). We observed in a proportion of cells that forskolin altered the sensitivity of CCh-evoked [Ca$^{2+}$], signals in such a way that apically confined [Ca$^{2+}$], signals were initiated from subthreshold CCh concentrations. In a variety of exocrine cells, the apical region is regarded as the “trigger zone” from which Ca$^{2+}$ waves are initiated (32, 50, 51). Conceptually, such a region could be established by the relative abundance of Ca$^{2+}$ release channels and/or expression of the most sensitive channels. Both of these criteria are satisfied in rat parotid acinar cells; immunocytochemical studies have reported that the extreme apical region is highly enriched in all InsP$_3$Rs (4, 18, 19). Furthermore, of the three InsP$_3$Rs expressed, the type II InsP$_3$R, which has been reported to be
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![Diagram](http://www.jbc.org/content/1347/11/6119/F9.large.jpg)

**Fig. 9.** PKA-mediated phosphorylation of InsP\(_3\)R by treatment with forskolin. A, parotid acini, metabolically labeled with \(^{32}\)PO\(_4\), were incubated with (+) or without (−) 10 \(\mu\)M forskolin for 10 min and samples were prepared as detailed under “Experimental Procedures.” Treatment with forskolin dramatically increased the intensity of a band representative of a ~250 kDa protein, indicative of phosphorylated InsP\(_3\)Rs. B, verification of Phospho-PKA substrate antibody in detecting phosphorylated proteins upon treatment of cells with (+) or without (−) 10 \(\mu\)M forskolin, 2 \(\mu\)M H-89, 30 \(\mu\)M Rp-cAMPS and/or 50 \(\mu\)M okadaic acid. C, a cell lysate prepared as detailed under “Experimental Procedures,” and protein samples run on a 7.5% SDS-polyacrylamide gel. Phosphorylated proteins were detected by Western blotting with the Phospho-PKA substrate antibody, C, detection of phosphorylated type II InsP\(_3\)Rs upon treatment of cells with (+) or without (−) 10 \(\mu\)M forskolin, 2 \(\mu\)M H-89 or 30 \(\mu\)M Rp-cAMPS. Phosphorylated proteins were immunoprecipitated with the Phospho-PKA substrate antibody, run on a 5% SDS-polyacrylamide gel and phosphorylated type II InsP\(_3\)Rs were detected by Western blotting with the CT\(_2\) antibody. * in A and C represent a secondary control whereby samples were treated identically but immunoprecipitating antibodies were omitted and represent nonspecific binding to protein-A beads.

The most sensitive to InsP\(_3\) (52, 53), is the most abundant InsP\(_3\)R type in parotid acinar cells (18). Collectively, this supports the idea that cAMP potentiates Ca\(^{2+}\) release from the most sensitive InsP\(_3\)R located in the apical region of the cell.

Ca\(^{2+}\)-induced Ca\(^{2+}\) release is important for the generation of both localized “spikes” and global increases in [Ca\(^{2+}\)]\(_i\) (4, 32), and it is conceivable that modulation of CICR could contribute to the observed cAMP-mediated potentiation. Although InsP\(_3\)R themselves possess all the properties to support CICR (54), there is an emerging body of evidence that RyRs are important for CICR in nonexcitable cells (5, 35, 55). Both InsP\(_3\)R and RyRs have been shown to contain putative consensus sequences for phosphorylation by PKA (56, 57) and in some cases phosphorylation results in enhanced release (58–60). However, in mouse parotid acinar cells, we found that inhibition of RyRs with high concentrations of ryanodine failed to significantly affect the CCh-evoked initial increase in [Ca\(^{2+}\)]\(_i\), consistent with the notion that the mechanism underlying this release primarily involves InsP\(_3\)R. When applied during a train of Ca\(^{2+}\) oscillations, however, ryanodine dramatically inhibited oscillations, similarly to previous studies (35, 61). Of particular interest, ryanodine significantly reduced the potentiation of the CCh-evoked [Ca\(^{2+}\)]\(_i\) response by forskolin (Fig. 6). An obvious explanation for this result is the direct phosphorylation of RyRs by PKA. However, we were unable to obtain any functional or biochemical evidence for this phosphorylation in parotid acinar cells. For example, various maneuvers designed to isolate Ca\(^{2+}\) release through RyR were not augmented by raising cAMP levels, and attempts to demonstrate direct phosphorylation of RyRs were unsuccessful. How can the effects of ryanodine on the initial PKA-potentiated release be reconciled with these data? One possibility is that the apparent contribution of RyRs after PKA treatment is simply the result of the enhanced release of Ca\(^{2+}\) by InsP\(_3\)R, resulting in an increased [Ca\(^{2+}\)]\(_i\) in the vicinity of RyR, thereby increasing the possibility of CICR. This implies that RyRs in parotid acinar cells act to amplify Ca\(^{2+}\) signals that originate from the primary trigger source, which is InsP\(_3\)R, but only so when a threshold [Ca\(^{2+}\)]\(_i\), (or microdomain of Ca\(^{2+}\)) is established. This is consistent with studies showing that RyR3 is the predominant RyR type expressed in mouse parotid acinar cells, albeit in relatively low abundance compared with skeletal muscle (62). Single-channel data indicate that RyR3 exhibit very low activity at [Ca\(^{2+}\)]\(_i\) lower than 1 \(\mu\)M, but are dramatically activated by [Ca\(^{2+}\)] above 1 \(\mu\)M (63). As such, RyRs would be functionally uncoupled at rest and only become activated by microdomains of Ca\(^{2+}\) created by Ca\(^{2+}\) released from neighboring InsP\(_3\)R.

Activation of PKA with Br-cAMP failed to evoke Ca\(^{2+}\) release from SL-O-permeabilized cells (Fig. FS), a result consistent with the lack of effect of forskolin in intact cells and in agreement with similar studies using imaging of calcium green C18-labeled permeabilized parotid acinar cells (20). In contrast, cAMP has been demonstrated to evoke Ca\(^{2+}\) release from static suspensions of permeabilized cells and microsomal vesicles of rat parotid acinar cells (18, 21–23), a result that was attributed to activation of RyRs. Of interest in these studies is the fact that cAMP elevation evoked a relatively small Ca\(^{2+}\) release (~35 nM) compared with InsP\(_3\) (~150 nM) (18, 21) and that this release occurred under conditions where basal [Ca\(^{2+}\)]\(_i\) was estimated to be as high as 431 nM (22). At these [Ca\(^{2+}\)]\(_i\), any RyRs would be expected to be in a sensitized state. The discrepancy between the present study and the aforementioned report underscores the difficulty in extrapolating averaged Ca\(^{2+}\) release from permeabilized cell suspensions to Ca\(^{2+}\) release in intact cells where resting [Ca\(^{2+}\)]\(_i\) is likely to be closer to ~100 nM. Alternatively, the presence of such a ryanodine-sensitive cAMP-dependent Ca\(^{2+}\) release pathway, not observed in this study, may simply be the result of species differences. As stated previously, mouse parotid acinar cells express predominantly RyR3 (62), whereas rat parotid acinar cells (revealed by reverse transcription-PCR) express predominantly RyR2 (21). Because RyR3 exhibit a lower threshold for activation by Ca\(^{2+}\) (63), any potentiation of Ca\(^{2+}\) release by cAMP would be amplified under conditions of elevated resting [Ca\(^{2+}\)]\(_i\) (18, 21, 22). Such species differences could also explain the lack of effect of caffeine in mouse acinar cells, as RyR3 are thought to be caffeine-insensitive (64, 65).

The lack of effect of ryanodine on InsP\(_3\)-evoked Ca\(^{2+}\) release in SL-O-permeabilized parotid acinar cells suggests that InsP\(_3\)R and RyRs are functionally uncoupled under these conditions, presumably as a result of high perfusion rates resulting in an effective infinite volume mimicking the cytoplasm. As such, microdomains of Ca\(^{2+}\) will likely fail to establish at
sufficiently high levels to evoke CICR. In addition, many cytosolic components such as mobile buffers, which may be important for CICR, will be lost in permeabilized cells. Thus, this system allowed us to study the functioning of InsP$_3$R$_i$s in isolation.

The data obtained indicate that the potentiation of CCH-evoked [Ca$^{2+}$]$_i$ signals is mediated through InsP$_3$Rs, because activation of PKA still revealed potentiation under conditions where RyRs were functionally uncoupled. In support of this, treatment with forskolin caused phosphorylation of type II InsP$_3$Rs; the weight of evidence presented suggests a PKA-mediated mechanism. There is an emerging consensus that PKA may regulate Ca$^{2+}$ release events directly at the level of the Ca$^{2+}$ release channel. Previous studies have shown that InsP$_3$Rs contain several consensus sequences for PKA-mediated phosphorylation (56) and can be phosphorylated both in vitro (66, 67) and in vivo (68, 69). However, there exists no clear consensus as to the physiological consequence of InsP$_3$R-type-specific phosphorylation in terms of Ca$^{2+}$ release. For example, phosphorylation of type I InsP$_3$R from cerebellum caused a decrease in Ca$^{2+}$ release (66, 70), whereas others have shown an increase in Ca$^{2+}$ release (71). In addition, phosphorylation of type III InsP$_3$R in pancreatic acinar cells results in reduced Ca$^{2+}$ release at low doses of InsP$_3$ (72), whereas phosphorylation of type II InsP$_3$R in liver enhances Ca$^{2+}$ release (58, 60). This latter observation is of particular interest to the present study, as the type II InsP$_3$Rs are the dominant form expressed in parotid acinar cells (18). Despite that in vitro phosphorylation studies revealed that type II InsP$_3$Rs were relatively poor substrates for PKA phosphorylation (69), the present study convincingly demonstrated that forskolin treatment causes PKA-mediated phosphorylation of type II InsP$_3$Rs in mouse parotid acinar cells.

In summary, PKA-mediated phosphorylation of type II InsP$_3$Rs is the predominant mechanism for the potentiation of Ca$^{2+}$ signaling in mouse parotid acinar cells. This largely explains the synergistic relationship between CAMP-raising agonists and ACh-evoked secretion in the parotid. These findings support the emerging consensus that phosphoregulation of InsP$_3$Rs is an important mechanism underlying the “shaping” of Ca$^{2+}$ signals, and thus have broad implications for the fidelity of Ca$^{2+}$-mediated processes.

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Phosphorylation of Inositol 1,4,5-Trisphosphate Receptors in Parotid Acinar Cells: A MECHANISM FOR THE SYNERGISTIC EFFECTS OF cAMP ON Ca2+ SIGNALING

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