Ryanodine and inositol trisphosphate receptors are differentially distributed and expressed in rat parotid gland

Xuejun ZHANG*, Jiayu WEN*, Keshore R. BIDASEE†, Henry R. BESCH, JR†, Richard J. H. WOJCIKIEWICZ‡, Bumsup LEE* and Ronald P. RUBIN*¹

*Department of Pharmacology and Toxicology, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, NY 14214, U.S.A.,

†Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202-5120, U.S.A., and ‡Department of Pharmacology, State University of New York Health Science Center at Syracuse, Syracuse, NY 13210, U.S.A.

The present study examines the cellular distribution of the ryanodine receptor/channel (RyR) and inositol 1,4,5-trisphosphate receptor (InsP₃R) subtypes in parotid acini. Using fluorescently labelled 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-propionic acid glyceryl-ryanodine (BODIPYTM-ryanodine) and confocal microscopy, RyRs were localized primarily to the perinuclear region (basal pole) of the acinar cell. Ryanodine, Ruthenium Red, cAMP and cADP ribose (cADPR) competed with BODIPY-ryanodine, resulting in a reduction in the fluorescence signal. However, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] did not alter the binding of BODIPY-ryanodine. Using receptor-subtype-specific antisera, InsP₃Rs (types I, II and III) were located predominantly in the apical pole of the parotid cell. The presence of these three subtypes was confirmed using reverse transcriptase PCR with RNA-specific oligonucleotide probes. Binding studies using a parotid cell-membrane fraction

identified and characterized RyRs and InsP₃Rs in terms of binding affinity (K_d) and maximum binding capacity (B_{max}) and confirmed that cADPR displaces ryanodine from its binding sites. Ruthenium Red and 8-Br-cADP-ribose blocked Ca²⁺ release in permeabilized acinar cells in response to cADPR and cAMP or forskolin, whereas Ins(1,4,5)P₃-induced Ca²⁺ release was unaffected. The localization of the RyRs and InsP₃Rs in discrete regions endow broad areas of the parotid cell with ligand-activated Ca²⁺ channels. The consequences of the dual activation of the RyRs and InsP₃Rs by physiologically relevant stimuli such as noradrenaline (norepinephrine) are considered in relation to Ca²⁺ signalling in the parotid gland.

Key words: cyclic ADP-ribose, cytochemical localization, InsP₃ receptor, parotid cell.

INTRODUCTION

There are two major mechanisms for mediating the release of intracellular Ca²⁺. One involves the action of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃]. This ligand, generated by phospholipase C-mediated polyphosphoinositide breakdown, exerts an action on a family of receptors that is located predominantly in the endoplasmic reticulum (ER) and which gates Ca²⁺ release from this organelle [1,2]. The receptors for Ins(1,4,5)P₃ (InsP₃Rs) comprise a family of three closely related subtypes (types I, II and III) that play a key role in regulating intracellular Ca²⁺.

The second mechanism utilizes a structurally related family of channels called the ryanodine receptor/channel (RyR) complex. The identification of ryanodine-sensitive Ca²⁺ stores was based in part on the fact that the plant alkaloid ryanodine binds to the RyR with high affinity and specificity and because the RyR is expressed as a result of the actions of such agents as cADP ribose (cADPR), cAMP, adenine nucleotides, caffeine, and Ruthenium Red [3–6]. Ryanodine binds to a family of receptors with subtypes found in skeletal muscle (type 1, RyR1) and cardiac muscle (type 2, RyR2), and also a widely distributed isoform that is found in, amongst others, smooth muscle (type 3, RyR3) [3,6,7]. In cardiac muscle the RyR-mediated mechanism for excitation–contraction coupling is Ca²⁺-induced Ca²⁺ release, whereas in skeletal muscle

the physiological importance of Ca²⁺-induced Ca²⁺ release is uncertain [7].

The parotid acinar cell is a morphologically and functionally polarized epithelial cell whose major function is to secrete amylase at the apical (luminal) pole and cations and fluid across the basolateral membrane. The agonist–receptor interactions that trigger these functional responses take place at the plasma membrane of the basal pole [8]. Our previous work showed that the concurrent activation of the cAMP- and phosphoinositide-Ca²⁺ pathways by the physiological neurotransmitter noradrenaline (norepinephrine) elicits optimal levels of Ca²⁺ mobilization and amylase secretion [9]. Additional studies using permeabilized parotid cells found that cAMP, but not Ins(1,4,5)P₃, mobilizes cellular Ca²⁺ by a ryanodine-sensitive mechanism [10] and that the RyR/Ca²⁺-release channel of parotid acinar cells is also modulated by cADPR, adenine nucleotides and calmodulin [5]. The postulate that cAMP and Ins(1,4,5)P₃ mobilize Ca²⁺ from different pools was supported by the findings that the mobilization of Ca²⁺ elicited by a maximal stimulating concentration of cAMP did not alter the Ca²⁺-releasing action of Ins(1,4,5)P₃ [10]. Moreover, heparin blocked the Ca²⁺-mobilizing action of Ins(1,4,5)P₃ but not that of cAMP, and the Ca²⁺-ATPase inhibitor thapsigargin only partially suppressed the Ca²⁺ response to cAMP but completely abolished the Ins(1,4,5)P₃

Abbreviations used: BODIPYTM-ryanodine, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-propionic acid glyceryl-ryanodine; cADPR, cADP-ribose; ER, endoplasmic reticulum; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; InsP₃R, Ins(1,4,5)P₃ receptor; RT-PCR, reverse transcriptase PCR; RyR, ryanodine receptor/channel.

¹ To whom correspondence should be addressed (e-mail rprubin@acsu.buffalo.edu).

response [10]. RyR expression was detected in isolated parotid cells by the fluorescently labelled ligand 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-propionic acid glyceryl-ryanodine, termed herein BODIPYTM-ryanodine [5,11], and by reverse transcriptase PCR (RT-PCR) [5]. Based upon these findings, it was proposed that Ca^{2+} stores in parotid acinar cells exist as two functionally distinct entities; one sensitive to $\text{Ins}(1,4,5)\text{P}_3$ and the other sensitive to ryanodine.

Since RyRs have been integrated into models of InsP_3 -induced Ca^{2+} signalling [12–14], specific knowledge of the cellular localization of the InsP_3R and RyR will help to determine the nature of the interactions between these two systems and even define their specific roles in signal transduction. Information about the cellular distribution of RyR has been very limited as compared with that of InsP_3R because the relatively low levels of RyR are more difficult to detect. In this article, we describe a differential pattern of distribution of the RyR and InsP_3R subtypes in parotid acini and further characterize their functional properties. The results demonstrate a broad distribution of Ca^{2+} -release channels throughout the parotid acinar cell and show that the RyR and InsP_3R subtypes are spatially and functionally distinct entities.

EXPERIMENTAL

Materials

Ruthenium Red and $\text{Ins}(1,4,5)\text{P}_3$ were purchased from Sigma (St. Louis, MO, U.S.A.). Fura-2, cADPR and ryanodine were from Calbiochem (San Diego, CA, U.S.A.) and 8-Br-cADPR was obtained from Molecular Probes (Eugene, OR, U.S.A.). $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (21 Ci/mmol) and $[^3\text{H}]\text{ryanodine}$ (62 Ci/mmol) were purchased from DuPont–NEN (Boston, MA, U.S.A.).

Permeabilization of cells

Parotid cells were prepared by sequential digestion with trypsin and collagenase of freshly isolated parotid glands from two rats as described previously [10]. For permeabilization, the cells were resuspended at a density of $(8\text{--}10) \times 10^6$ cells/ml and washed three times with a cytosol-like medium that had the following composition: 140 mM KCl/20 mM NaCl/20 mM Hepes/3 mM ATP/10 mM phosphocreatine/10 units/ml creatine phosphokinase (pH 7.2). After resuspension in the intracellular-like solution, 60 $\mu\text{g}/\text{ml}$ saponin was added and the cells were stirred at room temperature for 3–4 min. After permeabilization, the cells were centrifuged and resuspended in high- K^+ buffer that contained 10 μM antimycin, 5 μM oligomycin and 6 mM ATP (final concentration). The metabolic inhibitors and ATP-generating system were present to prevent responses from any residual intact cells and to ensure that the responses observed were entirely due to permeabilized cells.

Measurements of the cytosolic Ca^{2+} concentration

Cytosolic Ca^{2+} levels were analysed in permeabilized cells as described previously [10]. Briefly, fura-2 was added to a final concentration of 1 μM . After 5–8 min, the cells were counted and divided into three aliquots of an equal number of cells ($\approx 10^6$). The change in fluorescence of each sample (1.6 ml) was then monitored in a Model RF-5000 Shimadzu spectrofluorophotometer after exposure to various treatments. Each experiment was repeated at least three times on a different preparation. Peak $[\text{Ca}^{2+}]_i$ values that were measured ≈ 1 min after the stimulus was added are represented as means ($\pm$ S.E.M.) with basal values subtracted. Basal $[\text{Ca}^{2+}]_i$ averaged 366 ± 14 nM ($n = 26$).

Fluorescence microscopic analysis

Confocal fluorescence microscopy was conducted on parotid glands taken directly from Sprague-Dawley rats and frozen in liquid nitrogen using a fluorescent ryanodine derivative prepared by coupling an activated ester of BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) 493/503 to glyceryl ryanodine (BODIPY-ryanodine) as described previously [11]. Sections (5–10 μm) were mounted on egg albumin-coated slides and washed with Krebs Ringer solution. They were then incubated for 3 h in total darkness at 37 °C in Hepes-buffered Krebs Ringer solution (pH 7.4) plus 25 μM BODIPY-ryanodine alone or together with other agents enumerated in the Results section. After the sections were washed three times with Krebs Ringer solution to remove unbound ligand, they were viewed initially using epifluorescence optics of a Nikon upright Optiphot microscope. Confocal imaging was then performed with a laser-scanning microscopy system (MRC-1024; Bio-Rad, Hercules, CA, U.S.A.) configured with a Nikon microscope and a krypton/argon laser (488 nm). A $\times 60$ oil-immersion objective was first employed to provide an overall view, and then zoom $\times 2.64$ optics yielded a high-magnification view. The system was operated by a Compaq Pentium 100 computer and photographs were processed using a disublimation printer.

Immunofluorescence analysis of InsP_3Rs

Initially, 5–10- μm -thick frozen sections of rat parotid gland were fixed and permeabilized with 100% acetone for 3 min, and then air-dried. After being treated for 30 min with PBS containing 0.1% Triton X-100 and washed three times with PBS, the sections were blocked for 1 h with 5% BSA in PBS. For immunofluorescence localization of InsP_3R subtypes, the primary antibody reaction was performed using a 1:20 dilution of affinity-purified InsP_3R subtype-I, -II or -III rabbit polyclonal antisera [15] diluted in PBS containing 5% BSA plus 0.5% Tween-20 and incubated overnight at room temperature. After the incubation, the sections were washed three times with PBS containing 0.5% Tween-20, and then reacted in total darkness with secondary antibody conjugated to fluorescein. After the sections were washed three times with PBS containing Tween-20 and covered with Fluoro-Guard Antifade Reagent (Bio-Rad), they were viewed using confocal microscopy as described above. Specificity of immunofluorescence was determined by incubating frozen sections with secondary antibody alone before microscopic analysis.

Binding studies

To determine specific $[^3\text{H}]\text{ryanodine}$ and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding, a membrane fraction was isolated from a parotid acinar cell preparation generated from three rats as described previously [10]. The cells were then resuspended in an isolation buffer composed of 10 mM imidazole/280 μM PMSF/1.1 μM leupeptin (pH 7.4 at 4 °C), and homogenized by hand using a Teflon pestle. The homogenate was then centrifuged for 10 min at 10000 g and the pellet resuspended in fresh isolation buffer. The protein concentration of the membrane fraction was then determined using the method of Lowry et al. [16] and the membranes were stored at -80 °C prior to use.

To carry out $[^3\text{H}]\text{ryanodine}$ binding, parotid cell membranes (2 mg/ml) were incubated for 2 h at 37 °C in 200 μl of media containing various concentrations of $[^3\text{H}]\text{ryanodine}$ (0–34 nM) plus 500 mM KCl/20 mM Tris/HCl (pH 7.4)/200 μM CaCl_2 . Non-specific binding was estimated in the presence of 1 μM unlabelled ryanodine. As for $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding, mem-

branes (2 mg/ml) were incubated for 30 min at 4 °C in 200 µl of media containing various concentrations of [3 H]Ins(1,4,5) P_3 (0–30 nM) plus 100 mM KCl/25 mM Na $_2$ HPO $_4$ /20 mM NaCl/1 mM EDTA/1 % BSA (pH 8.3). Non-specific binding was determined in the presence of 30 µM unlabelled Ins(1,4,5) P_3 . For both [3 H]ryanodine and [3 H]Ins(1,4,5) P_3 analysis, bound and unbound ligand were separated by filtration through Whatman GF/C filters (0.45 µm) and the filter-bound radioactivity analysed by liquid-scintillation counting. Binding curves and $B_{\max}$ and K_d values were generated using Prism (GraphPad 2.0).

Western-blot analyses

To identify type-specific Ins P_3 Rs, cell lysates from isolated parotid cells were subjected to electrophoresis and subsequently transferred to a PVDF membrane. The membrane was blocked with PBS plus 7 % non-fat dry milk and 0.2 % Tween-20 and then incubated with affinity-purified Ins P_3 R subtype-I, -II or -III antisera [15], diluted 1:75, 1:150 and 1:75 respectively in PBS plus 7 % milk. The primary antibody reaction was carried out at 4 °C overnight. After immunoreactivity was visualized using peroxidase-conjugated secondary antibody, the blot was processed for enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A.) and exposed to X-ray film. To assess the relative abundance of each receptor subtype, the dilution of antibody was adjusted so that equal amounts of purified subtypes I, II or III receptor standards produced bands of approximately equal intensity [15]. Receptor-subtype immunoreactivity was quantified by analysing each band using a scanning densitometer and Molecular Analyst software (Bio-Rad).

mRNA analysis and RT-PCR

mRNA was isolated from rat parotid acinar cells using Trizol reagent (Gibco Life Technologies). First-strand cDNA was synthesized using a SuperScript II ribonuclease H⁻ RT kit (Gibco Life Technologies). Rat Ins P_3 R subtype-specific primers were designed according to the data base provided in the GenBank. The primers used were as follows: type-I sense, 5'-GAGAGAAAGCGCACGCCGAGAG-3' (92–113); type-I antisense, 5'-CATAGCTTAAGAGGCAGTCTC-3' (490–511); type-II sense: 5'-CGGGAATTCGGAGCTTCCAACCTCAAG-3' (1251–1269); type-II antisense, 5'-CACAAGCTTAGCTTCTTCACCGTGGTGG-3' (1604–1622); type III sense, 5'-GGCCGGAATTCAGAGAAGATCGCCGA-3' (377–391); and type-III antisense, 5'-GGACGAAGCTTCTTGCCCCGGTACTC-3' (900–914). Underlined sequences indicate restriction enzyme sites added to the specific sequences to aid in cloning and sequencing. Using these primers, Ins P_3 R-specific cDNAs were amplified by RT-PCR in a thermocycler (model 2400, Perkin-Elmer) using 3 units of *Taq* polymerase in a 50-µl reaction volume. In experiments that probed the type-I receptor, 2 units of *Pfu* DNA polymerase were substituted for *Taq*. PCR reactions were run for 35 cycles consisting of 1 min of denaturation at 95 °C, 1.5-min annealing at 55 °C and 60 °C with *Taq* and *Pfu*, respectively, and 2.5-min extension at 74 °C. The PCR products were analysed by electrophoresis on 1.8 % agarose gels and DNA was visualized by ethidium bromide staining.

RESULTS

Detection of RyR by fluorescence confocal microscopy

Since ryanodine is an invaluable tool for characterizing the RyR [17], the availability of the fluorescently labelled BODIPY-ryanodine affords a means to investigate the cellular localization

Figure 1 Confocal images demonstrating the fluorescence localization of ryanodine-binding sites in rat parotid gland

Representative images of acini labelled with (A) 25 µM BODIPY-ryanodine or (B) BODIPY-ryanodine plus 250 µM ryanodine as described in the Experimental section are shown. Scale bar: 10 µm.

of RyRs. Validation of the use of BODIPY-ryanodine to identify RyRs stems from the fact that the pharmacology of the inhibition of [3 H]ryanodine occupancy by BODIPY-ryanodine in skeletal muscle microsomes (IC_{50} 100 nM) is comparable with that observed with competing unlabelled ryanodine, although $\approx$ 10-fold less potent [5]. Acini exposed to BODIPY-ryanodine displayed a granular fluorescence signal throughout the cytoplasm that appeared to be associated with a cellular organelle or membrane (Figure 1A). The signal was most intense in the perinuclear region of the basal pole and gradually diminished towards the apical pole. In fact, in certain sections fluorescence was virtually undetectable in the distal apical pole (Figure 1A). Competition experiments to determine binding specificity showed that the fluorescence signal was reduced to undetectable levels by treating acini with a combination of BODIPY-ryanodine plus excess unlabelled ryanodine (Figure 1B).

Figure 2(A), which shows a pseudocolour representation of a parotid acinus, clearly illustrates that the highest levels of RyR expression were localized to the periphery of the basal pole; the expression of RyRs was markedly reduced in the apical pole and was undetectable in the nucleus. The pattern of distribution of RyRs corresponded to the density of the ER, which is concentrated in the basal pole and is only expressed sparsely in the apical pole [18]. Thus based upon the fluorescence pattern of BODIPY-ryanodine, it is likely that the RyRs are localized to intracellular organelles throughout the cytoplasm, most probably

Figure 2 The effects of RyR-interactive agents on BODIPY-ryanodine binding

Panels show the fluorescence intensity for acini exposed to: (A) 25 μ M BODIPY-ryanodine alone; or BODIPY-ryanodine plus (B) 100 μ M cADPR; (C) 1 mM cAMP; or (D) 250 μ M Ruthenium Red. The colour scale in the bar between (A) and (B) represents pseudocolour intensity levels according to an arbitrary scale. Scale bar, 10 μ m.

the ER. Using a green fluorescent protein fused to RyR, Bhat et al. [19] demonstrated that skeletal-muscle RyR expressed in Chinese hamster ovary cells was also localized to intracellular membranes, particularly in the perinuclear region.

Competition experiments were also carried out using various agents that interact purportedly with RyRs. cADPR and cAMP, which stimulate Ca^{2+} release via a ryanodine-sensitive mechanism [5,10], also markedly reduced the fluorescence signal produced by BODIPY-ryanodine (Figures 2B and 2C), as did Ruthenium Red, a RyR blocker (Figure 2D). The failure of $\text{Ins}(1,4,5)\text{P}_3$ to depress the signal (Figure 3) substantiates the notion that the InsP_3R and RyR are distinct entities.

Expression and localization of InsP_3R subtypes

Prior to assessing their cellular distribution, the identity of InsP_3R subtypes present in parotid cells was determined. Analysis of DNA fragments generated by RT-PCR revealed the presence of transcripts for subtypes I, II and III (Figure 4). To characterize the expression and the relative abundance of each subtype, immunoblot analysis was also carried out on cell lysates. Three immunoreactive bands were obtained by antibodies specific for type-I, -II and -III receptors, respectively (Figure 5). These bands co-migrated with those obtained using authentic type-I, -II and -III InsP_3R standards (results not shown). The data revealed that the type-II receptor was most abundant (30 ± 2 ng/100 μ g of total cell protein); type III was much less abundant (2.6 ± 0.8 ng); and type I was expressed in even lower amounts (0.4 ± 0.1 ng).

The relative abundance of the type-I, -II and -III receptors (their contribution to total InsP_3R immunoreactivity) was 1.0 ± 0.3 , 89 ± 3 and 10 ± 3 %, respectively ($n = 3-4$).

Using antisera specific to InsP_3R subtypes I, II and III, the expression and distribution of InsP_3Rs were explored in frozen sections of parotid glands. Immunofluorescence was observed in acini using the three type-specific antibodies (Figure 6); and again the type-II receptor appeared to be the most highly expressed (Figure 6B). Although each of the receptor subtypes was localized to the apical pole (Figure 6), the perinuclear region also expressed type-II and -III receptors (Figures 6B and 6C). Whereas type-III receptors were distributed at the apical pole and in regions in close proximity to or at the lateral plasma membrane (Figure 6C), none of the receptor subtypes were detected in the nucleus. However, the type-I receptor was expressed in the nuclei of ductal cells (results not shown).

Binding of [^3H]ryanodine and [^3H] $\text{Ins}(1,4,5)\text{P}_3$

Experiments to further characterize the RyR and InsP_3R population were also carried out by incubating parotid cell membranes with increasing amounts of radioligand. Scatchard analysis of a representative experiment depicting the [^3H]ryanodine-binding constants (K_d and B_{max}) is provided in Figure 7(A). Within the concentration range of 0–34 nM [^3H]ryanodine, the binding of radioligand was concentration-dependent and saturable, exhibiting an average K_d of 8.7 nM. This K_d value, which represents the affinity of ryanodine for the single isoform of RyR detected

Figure 3 Lack of effect of $\text{Ins}(1,4,5)\text{P}_3$ on BODIPY-ryanodine binding

Pseudocolor images of acini incubated with 25 μM BODIPY-ryanodine in the absence (A) and presence (B) of 25 μM $\text{Ins}(1,4,5)\text{P}_3$. Scale bar, 10 μm .

Figure 4 RT-PCR analysis of InsP_3R subtypes in parotid cells

RT cDNA (2 μg) from rat parotid cell total RNA was subjected to RT-PCR analysis using type-specific primers as described in the Experimental section. The PCR products were processed by electrophoresis and the cDNA was stained with ethidium bromide. Shown is the expression of transcripts for InsP_3R subtypes I (lane I), II (lane II) and III (lane III). The transcript for brain type-I RyR (BR) is also shown for comparison. M, molecular-size markers (300–900 bp).

by RT-PCR in rat parotid cells [5], is comparable with that previously obtained using mouse parotid microsomes [20]. Assuming that high-affinity ryanodine binding was measured and a single high-affinity binding site is present, the density of RyRs in rat parotid cells was estimated to be 82 fmol/mg of protein. When compared with mouse, rat parotid glands expressed 3.3 times fewer RyRs [20]. This disparity may be ascribed to species variability or to differences in the procedures used to isolate the subcellular fractions.

Figure 5 Immunoblot analysis of rat parotid cell lysates for InsP_3R subtypes

Cell lysates (20 μg for type II and 150 μg for types I and III) and InsP_3R standards (2 ng) were run on an SDS/polyacrylamide gel (6% gel), transferred on to PVDF membranes and probed with anti-type-I, -II or -III InsP_3R antisera (lanes 1, 2 and 3 respectively). Data shown are representative of three analyses of two different cell preparations.

Because cADPR was found to reduce markedly BODIPY-ryanodine fluorescence in intact acini, presumably by displacing the fluorescent ligand from ryanodine-binding sites, it was of interest to determine whether cADPR altered [^3H]ryanodine binding in the membrane fraction. Figure 8 shows that cADPR exhibited a concentration-dependent inhibition of radioligand binding, causing an almost complete inhibition at 13.5 μM . Interestingly, the concentration of cADPR that reduces [^3H]ryanodine binding by $\approx 50\%$ (0.4 μM) coincides with the EC_{50} for cADPR in enhancing Ca^{2+} release from permeabilized parotid cells (0.6 μM) [5].

Equilibrium binding-affinity assays were also used to determine the density of InsP_3Rs . Within the concentration range of 0–30 nM [^3H] $\text{Ins}(1,4,5)\text{P}_3$, the binding of radioligand to acinar cell membranes was concentration-dependent and saturable, exhibiting an apparent mean K_d of 7.6 nM and a B_{max} of 46 fmol/mg of protein. Scatchard analysis of a representative experiment depicting [^3H] $\text{Ins}(1,4,5)\text{P}_3$ binding is provided in Figure 7(B). Since all three $\text{Ins}(1,4,5)\text{P}_3$ subtypes have been identified in rat parotid cells, the apparent K_d represents the average of the affinities of $\text{Ins}(1,4,5)\text{P}_3$ for the three subtypes. Similarly, the B_{max} value represents the sum of the densities of the three subtypes. Still, assuming one $\text{Ins}(1,4,5)\text{P}_3$ -binding site on each receptor, taken collectively these data suggest that rat parotid cells express almost twice (1.78) as many RyRs as InsP_3Rs .

Effects of antagonists on Ca^{2+} release

Having obtained morphological evidence for the differential cellular distribution of the RyR and InsP_3R /channel and for the distinctive binding properties of the RyR, we extended our investigation using pharmacological analysis to strengthen the concept that the RyR and the InsP_3R are also distinct functional entities. The development of this concept was based upon our previous studies that characterized comprehensively the Ca^{2+} -mobilizing actions of $\text{Ins}(1,4,5)\text{P}_3$, cAMP and cADPR [5,10]. In the present study, the comparative actions of the RyR blocker Ruthenium Red on Ca^{2+} mobilization elicited by cADPR, cAMP and $\text{Ins}(1,4,5)\text{P}_3$ were examined in permeabilized parotid cells. The time course of a representative experiment using cADPR as the agonist is shown in Figure 9(A) and a quantitative assessment is provided in Figure 9(B). Ruthenium Red produced a concentration-dependent inhibition of Ca^{2+} release in response to submaximal concentrations of cADPR and cAMP, but had no effect on Ca^{2+} release elicited by $\text{Ins}(1,4,5)\text{P}_3$.

Figure 6 Localization of InsP_3R subtypes in parotid acini

Immunofluorescence imaging by confocal microscopy after staining with type-I-, -II- and -III-specific antibodies are shown in (A), (B) and (C) respectively. Scale bar, 10 μm .

We next examined whether the synthetic cADPR analogue 8-Br-cADPR is capable of modifying Ca^{2+} mobilization from permeabilized cells. Attempts to demonstrate a blockade of cAMP-induced Ca^{2+} release by 8-Br-cADPR yielded variable results, possibly due to the high concentrations of cAMP (100 μM) relative to antagonist (10 μM) that were employed. Since we have demonstrated previously that Ca^{2+} release from intact and permeabilized parotid cells can be regulated by endogenously generated cAMP [5], we utilized forskolin to elevate cAMP levels. The time course of a representative experiment showing the ability of 8-Br-cADPR to reduce the Ca^{2+} -mobilizing action of forskolin is given in Figure 10(A) and a quantitative assessment is shown in Figure 10(B). The enhancement of Ca^{2+} release induced by cADPR was also reduced by 8-Br-cADPR

Figure 7 Scatchard analysis of the equilibrium binding of $[^3\text{H}]$ ryanodine (A) and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (B) to a membrane fraction of parotid acinar cells

The x and y intercepts yield estimates of total receptor concentration (B_{max}) and ligand affinity (K_D), respectively. Data shown are from a representative experiment performed in duplicate that was repeated on four different preparations (see the text for details).

Figure 8 cADPR inhibits $[^3\text{H}]$ ryanodine-specific binding

Incubations of a membrane fraction (100 $\mu\text{g}/\text{ml}$) were carried out for 2 h at 37 $^{\circ}\text{C}$ in the presence of 3.4 nM $[^3\text{H}]$ ryanodine and varying concentrations of cADPR as indicated. Non-specific binding was determined by incubating membranes in the presence of 50 μM cADPR. Values represent means $\pm$ S.E.M. of three independent experiments each carried out in duplicate.

(Figure 10B). The inhibitory action of the cADPR antagonist was selective in that it failed to alter the Ca^{2+} response to $\text{Ins}(1,4,5)\text{P}_3$ (Figure 10B).

Figure 9 Effects of Ruthenium Red on cADPR-, cAMP- and $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} mobilization

In (A) a concentration-dependent increase in Ca^{2+} release elicited by cADPR (●) and its inhibition by 10 μM Ruthenium Red (○) in permeabilized parotid cells are shown. The lack of effect of Ruthenium Red on the response to 3 μM $\text{Ins}(1,4,5)\text{P}_3$ (Ip_3) is also illustrated. Each trace is representative of at least five traces obtained from four different preparations. In (B) permeabilized cells were treated with (5 or 20 μM ; hatched and cross-hatched bars respectively) or without (open bars) Ruthenium Red for 5 min followed by cADPR (1 μM), cAMP (100 μM) or $\text{Ins}(1,4,5)\text{P}_3$ (3 μM). Values (means $\pm$ S.E.M. of three experiments) represent peak Ca^{2+} release with basal values subtracted ($\Delta[\text{Ca}^{2+}]$). Ruthenium Red (5 and 20 μM) alone increased basal Ca^{2+} release by 14 ± 3 and 30 ± 6 nM, respectively ($n = 8-9$). *Significantly different from corresponding control group ($P < 0.05$).

It has been suggested that cADPR analogues serve as competitive inhibitors by binding to the cADPR receptor without activating it [4]. However, in our hands, 10 μM 8-Br-cADPR and 8-amino-cADPR raised basal Ca^{2+} release by an average of 22 ± 3 nM ($n = 10$) and 28 ± 5 nM ($n = 4$), respectively. Because these cADPR antagonists also act as partial agonists in parotid cells, one cannot rule out the possibility that their inhibitory effects may at least in part be attributed to the depletion of ryanodine-sensitive Ca^{2+} stores.

DISCUSSION

Because agonist-induced Ca^{2+} signalling in non-excitable cells involves a high degree of spatio-temporal complexity [13], comparative analysis of the distribution patterns of the RyR and InsP_3R subtypes could prove crucial for elucidating the sequence of events associated with Ca^{2+} signalling. The present investigation, which demonstrates the pattern of distribution of BODIPY-ryanodine fluorescence in intact acini, fills an important gap in our knowledge. In our previous study the loss of cell polarity accompanying the disruption of the acinar configuration ruled against optimal localization of a fluorescent signal to a specific cytoplasmic component of dispersed parotid cells [5]. In intact acini the basal pole expressed the highest levels of RyRs,

Figure 10 Effects of 8-Br-cADPR on cADPR-, forskolin- and $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} mobilization

In (A) a concentration-dependent increase in forskolin (FSK)-stimulated Ca^{2+} mobilization (●) and its inhibition by 10 μM 8-Br-cADPR (○) in permeabilized parotid cells are shown. Each trace is representative of at least five traces obtained from four different preparations. Each arrow indicates the addition of FSK to control cells and those treated with 8-Br-cADPR. In (B) permeabilized cells were treated with (hatched bars) or without (white bars) 10 μM 8-Br-cADPR for 5 min followed by cADPR (1 μM), FSK (10 μM) or $\text{Ins}(1,4,5)\text{P}_3$ (Ip_3 ; 3 μM). Values are means $\pm$ S.E.M. of three independent experiments with basal values subtracted. *Significantly different from corresponding control group ($P < 0.05$).

where the ER is most concentrated [18,21], and the expression of receptors decreased through the apical pole where the ER is sparsely expressed, thus suggesting the ER as the locus of RyR expression. RyR expression has also been detected by immunofluorescence in the submandibular (salivary) gland, but in contrast with our findings its localization was confined to the luminal pole and basolateral membrane [22]. The divergent findings may be tissue related or due to differences in experimental procedures, i.e. use of BODIPY-ryanodine versus anti-RyR antibody.

The documentation of specific binding sites for ryanodine was provided by the ability of unlabelled ryanodine and Ruthenium Red to reduce markedly the fluorescence signal by displacing BODIPY-ryanodine. The specificity of the binding to RyR was also verified by the inability of $\text{Ins}(1,4,5)\text{P}_3$ to displace BODIPY-ryanodine and further substantiated by binding experiments using [^3H]ryanodine.

Functional studies are pivotal in supporting the claim that the BODIPY-ryanodine binding studies bear a relation to physiological function. In accord with the cytochemical studies which showed that Ruthenium Red was able to compete with BODIPY-ryanodine for RyR occupancy, permeabilized parotid cells pre-

treated with Ruthenium Red failed to mobilize Ca^{2+} in response to cAMP and cADPR. Furthermore, the findings that $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release was not blocked by Ruthenium Red and that BODIPY-ryanodine fluorescence was not altered by $\text{Ins}(1,4,5)\text{P}_3$ argue in favour of the view that RyR- and InsP_3 -mediated Ca^{2+} release originate from independently operated stores. The supposition that cAMP and $\text{Ins}(1,4,5)\text{P}_3$ mobilize Ca^{2+} from different pools is substantiated by our previous findings that Ca^{2+} release produced by a maximal stimulating concentration of cAMP did not depress $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release and that ryanodine, thapsigargin and heparin exhibited distinct pharmacological profiles with regard to their actions on cAMP- and $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores [5,10]. The inability of ryanodine to displace $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ from its binding sites in a membrane fraction of parotid cells also supports this conclusion (results not shown). The differential cellular distribution and diverse mechanisms of regulation for the RyRs and InsP_3 Rs, as reported herein, imply functional specificity and may provide a basis for ultimately resolving the various components responsible for Ca^{2+} signalling.

Our findings that cAMP and cADPR inhibited BODIPY-ryanodine occupancy and that cADPR caused a pronounced reduction in $[^3\text{H}]\text{ryanodine}$ binding suggest that adenine nucleotides interact with the RyR complex, rather than with an accessory protein such as a protein kinase [4,23]. Moreover, the fact that the cADPR concentration that displaces 50% of the radiolabelled ligand in a membrane fraction closely approximates to the EC_{50} for cADPR in causing RyR-mediated Ca^{2+} mobilization from permeabilized parotid cells is consistent with the view that the RyR is a primary target of cADPR action. Whereas adenine nucleotides such as ATP, cAMP and cADPR probably bind to the same site on the RyR (adenine-nucleotide-binding site) [23–25], qualitative differences appear to exist in the properties of $[^3\text{H}]\text{ryanodine}$ binding to various preparations, which may depend upon the nature of the RyR. Thus in skeletal-muscle sarcoplasmic reticulum adenine nucleotides enhance $[^3\text{H}]\text{ryanodine}$ binding, whereas in cardiac sarcoplasmic reticulum, adenine nucleotides were reported to have little or no effect on ryanodine binding [3]. But it is most relevant to note that, in rat brain, adenine nucleotides have a biphasic effect on ryanodine binding, enhancing binding at low concentrations ($< 1 \text{ mM}$) and inhibiting binding at higher concentrations [26]. Based upon $[^3\text{H}]\text{ryanodine}$ -binding studies, a co-operative interaction between the ryanodine-recognition site and the adenine-nucleotide-binding site has been proposed [26]. Although our findings do not rule out the possibility that cADPR binds to a separate accessory protein, which in turn interacts with the RyR [23], there appear to be sufficient grounds for suspecting that the competition between ryanodine and adenine nucleotides in parotid cells involves a negative allosteric interaction.

The interpretation of our present findings in mechanistic terms must be guarded in light of our present incomplete understanding of how various convergent factors such as Ca^{2+} , Mg^{2+} , calmodulin and adenine nucleotides regulate the RyR. Still, our previous work provides sufficient grounds for advocating that there may be pitfalls associated with the view that agents which act on the muscle RyR isoforms regulate parotid RyR activity by a similar mechanism. Thus the characteristic property of ryanodine to open the RyR in low concentrations and block the channel in higher concentrations is not observed in permeabilized parotid cells [5]. Moreover, the well-established ability of caffeine to markedly stimulate Ca^{2+} release from muscle cannot be duplicated in the parotid acinar cell [5]. These findings, taken together with preliminary Western-blot analysis of parotid cell membranes using antibodies raised against type-I, -II and -III RyRs, which

failed to reveal protein bands that have electrophoretic mobility patterns similar to that of the RyR (K. R. Bidasee, unpublished work), are compatible with the existence of a novel RyR isoform. Indeed, we have recently found by partial sequence analysis that the RyR expressed in rat parotid cells is similar to a truncated RyR type I expressed in rabbit brain [5,27]. Like the rabbit brain RyR [27], the parotid receptor appears to possess the C-terminal (channel) component but lacks the caffeine-sensitive N-terminal portion [5,19]. Because of their unique structures, the truncated RyR type I in brain and parotid gland may perform its function as a Ca^{2+} -release channel by a mechanism that is divergent from that of authentic skeletal-muscle RyR [28]. To clarify this issue, further studies are now underway to gain further insight into the structure of the RyR in parotid acinar cells.

It was also important to assess the cellular distribution of the InsP_3 Rs in order to determine whether the two systems for Ca^{2+} release are co-localized in parotid cells or are spatially distinct. In contrast with the RyRs that predominate in the basal pole, immunolocalization studies showed that the three InsP_3 R subtypes (I, II and III) identified by RT-PCR and immunoblotting were enriched in the apical pole of the cell. The three InsP_3 R subtypes previously detected in exocrine pancreas and submandibular (salivary) glands were likewise localized in proximity to the apical (luminal) membrane [22,29]. Because $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} release is closely linked to the ER [30], the immunofluorescence data that identify the apical pole as a major site of the InsP_3 R and therefore a primary locus of Ca^{2+} signalling must be reconciled with morphological data revealing that apical ER is sparsely expressed in the resting parotid cell [18,21]. Whereas Ca^{2+} -containing zymogen granules predominate in the apical pole, they do not appear to be responsible for any major contribution to Ca^{2+} signalling in exocrine acinar cells, including parotid cells [29,31]. Since each InsP_3 R subtype may perform unique cellular functions, selective activation of individual subtypes by agonists may provide one level of control of Ca^{2+} signalling. Our findings using both immunofluorescence and biochemical analysis, which showed that parotid cells express a predominant amount of type-II receptor and much lesser amounts of type-I and -III receptors, suggest that the type-II receptor plays a key role in Ca^{2+} signalling in this epithelial cell. The comparative $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ - and $[^3\text{H}]\text{ryanodine}$ -binding studies, which confirmed the presence of specific high-affinity binding sites for both ryanodine and $\text{Ins}(1,4,5)\text{P}_3$, determined that the InsP_3 Rs were somewhat less abundant than the RyRs. However, the number of InsP_3 Rs and RyRs does not appear to be correlated with the extent of Ca^{2+} mobilization since $\text{Ins}(1,4,5)\text{P}_3$ is at least 2–3 times more effective in promoting Ca^{2+} release from permeabilized cells than agents such as cAMP or cADPR that utilize ryanodine-sensitive stores. Thus in contrast with the more diffuse distribution of RyR, the clustering of InsP_3 R in discrete areas of the parotid cell [32], taken together with the differential ability of $\text{Ins}(1,4,5)\text{P}_3$ to bind to the different isoforms [33], may result in localized domains of high levels of cytoplasmic Ca^{2+} .

The association of the type-III InsP_3 R with the lateral plasma membrane or with the ER juxtaposed with the plasma membrane as observed in the parotid gland, and previously reported in the submandibular gland [22], may have additional functional significance by identifying a system in which the InsP_3 R/channel mediates the passage of Ca^{2+} to neighbouring cells via gap junctions. Such a mechanism may co-ordinate and amplify changes in cytoplasmic Ca^{2+} that occur between adjacent cells of the acinus [34]. This idea is not without precedent since the association of gap junctions with ER cisternae described earlier in parotid cells was envisioned to control junctional permeability

to Ca^{2+} [35]. In addition, an $\text{Ins}(1,4,5)\text{P}_3$ -sensitive site closely associated with the plasma membrane may be responsible for the domains of high Ca^{2+} that can be measured in the sub-plasmalemmal space [36].

Whereas details concerning mechanisms involved in Ca^{2+} signalling in parotid cells are still incomplete, the present work indicates that the distribution patterns of the InsP_3R subtypes and RyRs are complementary rather than coincident in the sense that together they endow broad areas of the parotid cell with ligand-activated Ca^{2+} channels. Although recent studies indicate that the parotid acinar cell stimulated by carbachol behaves like the pancreatic acinar cell [22], in that the Ca^{2+} wave is initiated at the apical pole and spreads toward the basal pole [31,37], the fact that parotid cells possess muscarinic (cholinergic), α -adrenergic and tachykinin (substance P) receptors that modulate $\text{Ins}(1,4,5)\text{P}_3$ levels, and β -adrenergic receptors which regulate cAMP synthesis, permits a widespread expression of Ca^{2+} signalling. Because the enhancement of sympathetic neurotransmission in the parotid gland is associated with the concurrent activation of α - and β -adrenergic receptors [38], the convergence of the α - and β -adrenergic signals should generate multiple Ca^{2+} waves that are consolidated into an integrated response to regulate amylase secretion at the apical pole and salt and water secretion at the basolateral membrane. Despite the fact that the relative contributions of InsP_3R and RyR to Ca^{2+} signalling may vary according to a given cell type, establishing a better understanding of the sequence of co-ordinated events involved in the regulation of intracellular Ca^{2+} in the parotid gland should enhance our knowledge of Ca^{2+} signalling in non-excitable cells.

These studies were supported by the National Institutes of Health research grants DE-059654 and R29 DK4914 and by the Showalter Trust. We thank Dr. Robert Summers of the Department of Anatomical Sciences and Cell Biology, State University of New York at Buffalo, Buffalo, NY, U.S.A. for his expert help in preparing the confocal images.

REFERENCES

- Joseph, S. K. (1996) *Cell Signal* **8**, 1–7
- Yoshida, Y. and Imai, S. (1997) *Jpn. J. Pharmacol.* **74**, 125–137
- Coronado, R., Morrisette, J., Sukhareva, M. and Vaughan, D. M. (1994) *Am. J. Physiol.* **266** (Cell Physiol. **35**), C1485–C1504
- Lee, H. C. (1997) *Physiol. Rev.* **77**, 1133–1164
- Zhang, X., Wen, J., Bidasee, K. R., Besch, Jr., H. R. and Rubin, R. P. (1997) *Am. J. Physiol.* **273** (Cell Physiol. **42**), C1306–C1314
- Meissner, G. (1994) *Annu. Rev. Physiol.* **56**, 485–508
- Sutko, J. L. and Airey, J. A. (1996) *Physiol. Rev.* **76**, 1027–1071
- Hand, A. R. (1990) in *Ultrastructure of the Extraparietal Glands of the Digestive Tract* (Riva, A. and Motta, P. M., eds.), pp. 1–17, Kluwer Academic Publishers, Dordrecht
- McKinney, J. S., Desole, M. S. and Rubin, R. P. (1989) *Am. J. Physiol.* **257** (Cell Physiol. **26**), C651–C657
- Rubin, R. P. and Adolf, M. A. (1994) *J. Pharmacol. Exp. Ther.* **268**, 600–606
- Bidasee, K. R. and Besch, Jr., H. R. (1998) *J. Biol. Chem.* **273**, 12176–12186
- Berridge, M. J. (1993) *Nature (London)* **361**, 315–325
- Bootman, M. D. and Berridge, M. J. (1995) *Cell* **83**, 675–678
- Thorn, P., Gerasimenko, O. and Petersen, O. H. (1994) *EMBO J.* **13**, 2038–2043
- Wojcikiewicz, R. J. H. (1995) *J. Biol. Chem.* **270**, 11678–11683
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Sutko, J. L., Airey, J. A., Welch, W. and Ruest, L. (1997) *Pharmacol. Rev.* **49**, 53–98
- Amsterdam, A., Ohad, I. and Schramm, M. (1969) *J. Cell Biol.* **41**, 753–773
- Bhat, M. B., Zhao, J., Zang, W., Balke, C. W., Takeshima, H., Wier, W. G. and Ma, J. (1997) *J. Gen. Physiol.* **110**, 749–762
- DiJulio, D. H., Watson, E. L., Pessah, I. N., Jacobson, K. L., Ott, S. M., Buck, E. D. and Singh, J. C. (1997) *J. Biol. Chem.* **272**, 15687–15696
- Chiarenza, A. P., Sanz, E. G., Vermouth, N. T., Aoki, A. and Bellavia, S. L. (1989) *Anat. Embryol.* **179**, 497–501
- Lee, M. G., Xu, X., Zeng, W., Diaz, J., Wojcikiewicz, R. J. H., Kuo, T. H., Wuytack, F. L., Racymaekers, L. and Muallem, S. (1997) *J. Biol. Chem.* **272**, 15765–15770
- Sitsapesan, R., McGarry, S. J. and Williams, A. J. (1995) *Trends Pharmacol. Sci.* **16**, 386–391
- Meissner, G. and Henderson, J. S. (1987) *J. Biol. Chem.* **262**, 3065–3073
- McGarry, S. J. and Williams, A. J. (1994) *J. Membrane Biol.* **137**, 169–177
- Zimanyi, I. and Pessah, I. N. (1991) *Brain Res.* **561**, 181–191
- Takeshima, H., Nishimura, S., Nishi, M., Ikeda, M. and Sugimoto, T. (1993) *FEBS Lett.* **322**, 105–110
- Furuichi, T., Kohda, K., Miyawaki, A. and Mikoshiba, K. (1994) *Curr. Opin. Neurobiol.* **4**, 294–303
- Yule, D. I., Ernst, S. A., Ohnishi, H. and Wojcikiewicz, R. J. H. (1997) *J. Biol. Chem.* **272**, 9093–9098
- Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) *Physiol. Rev.* **74**, 595–636
- Liu, P., Scott, J. and Smith, P. M. (1998) *Biochem. J.* **330**, 847–852
- Wilson, B. S., Pleiffer, J. R., Smith, A. J., Oliver, J. M., Oberdorf, J. A. and Wojcikiewicz, R. J. H. (1998) *Mol. Biol. Cell* **9**, 1465–1478
- Newton, C. L., Mignery, G. A. and Sudhof, T. C. (1994) *J. Biol. Chem.* **269**, 28613–28619
- Yule, D. I., Stuenkel, E. and Williams, J. A. (1996) *Am. J. Physiol.* **271** (Cell Physiol. **40**) C1285–C1294
- Dunn, J. and Revel, J.-P. (1984) *Cell Tissue Res.* **238**, 589–594
- Marsault, R., Murgia, M., Pozzan, T. and Rizzuto, R. (1997) *EMBO J.* **16**, 1575–1581
- Tojyo, Y., Tanimura, A. and Matsumoto, Y. (1997) *Cell Calcium* **22**, 455–462
- Thulin, A. (1976) *Acta Physiol. Scand.* **96**, 506–511

Received 25 November 1998/11 February 1999; accepted 23 March 1999