Nitric oxide and cGMP activate Ca^{2+} -release processes in rat parotid acinar cells

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We characterized the enzymic properties of ADP-ribosyl cyclase in rat parotid acinar cells by using a fluorescence technique. ADP-ribosyl cyclase is capable of synthesizing the Ca^{2+} -mobilizing nucleotide cADP-ribose (cADPR) from NAD^+ and has previously been shown to be regulated by cGMP via a cGMP-dependent protein kinase (G kinase). We therefore investigated whether NO/cGMP-activated pathways are present in rat parotid acinar cells and whether NO/cGMP signalling exerts control over cellular Ca^{2+} signalling processes. Our results showed that stimulation of acinar cells with adrenaline, isoproterenol, substance P and NO resulted in a rise in the [cGMP]. In addition, NO induced a release of Ca^{2+} from intracellular ryanodine-

sensitive stores via a cGMP/G-kinase-mediated process. Thus our data reveal that a rise in [cGMP], caused by either neurotransmitter or NO activation, activates a G kinase, which in turn controls Ca^{2+} release from ryanodine-sensitive stores. Since parotid acinar cells possess ADP-ribosyl cyclase activity, we propose a model in which cADPR is the link between NO/cGMP signalling pathways and release of Ca^{2+} from ryanodine-sensitive stores.

Key words: ADP-ribosyl cyclase, G kinase, intracellular free Ca^{2+} concentration, stimulus–secretion coupling.

INTRODUCTION

NO, synthesized by the enzyme nitric oxide synthase, has been shown to be a ubiquitous intracellular messenger that leads to a variety of signalling processes. NO is thought to play an important role in pathways that involve regulation of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Its role in secretory processes is currently being investigated, since nitric oxide synthase has been discovered in nerve endings surrounding glandular tissue as well as in acinar and ductal cells from pancreatic and salivary glands [1,2].

NO mediates many of its effects by activation of soluble guanylate cyclases, thus leading to an increase in cGMP synthesis. In sea urchin eggs, the NO-induced cGMP production initiates a cascade of signalling events, the first step being activation of a cGMP-dependent kinase (G kinase). The kinase phosphorylates and activates ADP-ribosyl cyclase, which in turn synthesizes the Ca^{2+} -mobilizing nucleotide cADP-ribose (cADPR), thereby leading to release of Ca^{2+} from ryanodine-sensitive Ca^{2+} stores [3]. A product of NAD^+ metabolism, cADPR was discovered by Clapper et al. [4] in extracts from sea urchin eggs and was shown to release Ca^{2+} from intracellular stores distinct from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive stores. cADPR has since been suggested as an endogenous ligand for the ryanodine receptor (RyR) [5]. In permeabilized rat parotid and lacrimal acinar cells, cADPR releases Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -insensitive stores [6,7]. This cADPR-induced Ca^{2+} release is inhibited by 100 μM ryanodine, confirming that the RyR is involved in the Ca^{2+} -release process.

The aim of this study was to investigate whether compounds that cause a rise in the intracellular [cGMP], such as agonists and NO, also modulate the $[\text{Ca}^{2+}]_i$ in rat parotid acinar cells. We also investigated the possibility that such a modulation of $[\text{Ca}^{2+}]_i$ might be mediated by a pathway similar to the one described for sea urchin eggs [3] by testing the importance of G kinase and ryanodine. Finally, we characterized the ADP-ribosyl cyclase activity in these cells, thus supporting the hypothesis of a cGMP/G kinase/cADPR pathway, although not providing conclusive evidence.

MATERIALS AND METHODS

Materials

Collagenase was from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). Caged cGMP and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were from Calbiochem (San Diego, CA, U.S.A.), Rp-8-(4-chlorophenylthio)guanosine-3',5'-cyclomonophosphorothionate (Rp-8-pCPT-cGMPS) was from BioLog (Bremen, Germany) and ryanodine was from Alomone Labs (Jerusalem, Israel). 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), CHAPS, nicotinamide guanine dinucleotide (NGD⁺), NAD^+ , hyaluronidase, substance P, adrenaline, isoproterenol and phenylephrine were purchased from Sigma (St. Louis, MO, U.S.A.) and fura 2 acetoxymethyl ester (fura 2/AM) was from Molecular Probes (Eugene, OR, U.S.A.). The soluble CD38 was a generous gift from Dr T. Katada and Dr M. Hara-Yokoyama (University of Tokyo, Tokyo, Japan).

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; cADPR, cADP-ribose; cGDP, cGDP-ribose; G kinase, cGMP-dependent protein kinase; cGMP-TME, cGMP tyrosyl methyl ester; RyR, ryanodine receptor; NGD⁺, nicotinamide guanine dinucleotide; PCA, perchloric acid; SNAP, *S*-nitroso-*N*-acetylpenicillamine; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; fura 2/AM, fura 2 acetoxymethyl ester; PEG, polyethylene glycol; Rp-8-pCPT-cGMPS, Rp-8-(4-chlorophenylthio)guanosine-3',5'-cyclomonophosphorothionate.

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The following materials were used for the cGMP RIA. Assay buffer, 10 mM sodium acetate/acetic acid buffer, pH 4.75; γ -globulin buffer, 100 mg of human γ -globulin fraction II (Fluka, Switzerland) dissolved in 100 ml of 50 mM sodium acetate/acetic acid buffer, pH 4.75; cGMP antiserum, cGMP antiserum (Calbiochem) diluted 1:3000 in γ -globulin buffer; second antibody on magnetic beads, Biomag goat anti-rabbit IgG (PerSeptive Diagnostics, Cambridge, MA, U.S.A.); 12% (w/v) polyethylene glycol (PEG), PEG 6000 (Merck, Darmstadt, Germany) dissolved in 50 mM sodium acetate/acetic acid buffer, pH 6.2; ^{125}I -labelled cGMP tyrosyl methyl ester (cGMP-TME), ^{125}I -cGMP-TME (Linco Research, St. Charles, MO, U.S.A.) dissolved in γ -globulin buffer; and 5 μM cGMP stock solution, cGMP (Calbiochem) dissolved in water.

Preparation of acini

All experiments were performed on parotid acini from male Wistar rats weighing approx. 130 g. Acini were isolated by a procedure using collagenase and hyaluronidase digestion as described previously [8] with the following modifications. The rats were killed by cervical dislocation and the parotid glands were extirpated and transferred to ice-cold RPMI 1640 medium containing 1.2 mM EGTA and 5 mM Hepes (pH 7.3). Fat, connective tissue and lymph nodes were removed and the glands were transferred to RPMI 1640 containing 75 units/ml collagenase, 153 units/ml hyaluronidase and 5 mM Hepes (pH 7.3, 37 °C). The glands were cut into pieces and incubated in the digestion medium for 45 min in a shaking water bath at 37 °C under continuous equilibration with 5% CO_2 /95% O_2 . The acini were dispersed by aspiration through polypropylene pipettes with orifice diameters of 2.5 and 1.7 mm and filtered through a 150 μm nylon cloth (Nytex). Acini were sedimented by centrifugation through RPMI 1640 medium with 1% serum albumin (1.5 min, 60 g) followed by one wash in the same medium. The acini were then resuspended in the medium of choice for the given type of experiment.

Preparation of acinar cell extracts and measurement of ADP-ribosyl cyclase activity

Acini were isolated as described above, washed in Ca^{2+} -free Krebs–Ringer solution (142 mM Na^+ /5 mM K^+ /1 mM Mg^{2+} /123 mM Cl^- /20 mM HCO_3^- /3 mM $\text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-}$ /5 mM Hepes, pH 7.3) and the cells were lysed in a hypo-osmotic solution (20 mM Tris/HCl, pH 7.0). To solubilize membrane-associated proteins, 5 mM CHAPS was added and the solution was agitated for 1 h at 4 °C, followed by centrifugation at 12000 g for 4 min. The supernatant containing the extracted enzymes was used for the experiments.

Enzyme extract was transferred to a cuvette and the initial fluorescence increase after adding NGD^+ directly into the cuvette was monitored using a Perkin-Elmer LS-5B fluorescence spectrophotometer. All experiments were performed at room temperature with an excitation wavelength of 300 nm and emission measured at 410 nm. In experiments where incubations were made with both NAD^+ and NGD^+ , NAD^+ was added directly into the cuvette immediately before the NGD^+ .

HPLC isolation of the incubation product

CD38 (100 ng/ml) and parotid enzyme extracts were incubated with 10 μM NGD^+ for 1–2 h. The product obtained from the incubation was isolated by HPLC after perchloric acid (PCA) precipitation of proteins (0.4 M PCA) and KOH neutralization.

A Mono Q HR 5/5 column (Pharmacia Biotechnology AB, Uppsala, Sweden) was used for separating the nucleotides. The flow rate was maintained at 1.5 ml/min and the nucleotides were eluted with trifluoroacetic acid using the following 0–100% gradient for solvent B (where solvent B is 60 mM trifluoroacetic acid and solvent A is water): solvent B was held at 0% for 1 min, increased linearly to 14% from 1 to 2 min, increased linearly to 17% from 2 to 12 min, increased linearly to 100% from 12 to 13 min and held at 100% until 22 min. Between runs the column was washed with 1 M trifluoroacetic acid. The eluate was collected in fractions of 1 ml by an automatic fraction collector.

Measurements of intracellular [cGMP]

Acini isolated as described above were resuspended in RPMI 1640 medium and stimulated by adding agonist from a stock solution directly into the suspension, followed by mixing. Samples were taken either before stimulation or at appropriate times after stimulation and the reaction was stopped for each sample by immediate and vigorous mixing with ice-cold PCA at a final concentration of 0.4 M PCA, followed by KOH neutralization.

The cGMP content of each sample was determined by RIA following a modification of the procedure provided with the cGMP antiserum by Calbiochem. Standards in the range 0–5 nM were prepared freshly prior to each assay by diluting a 5 μM cGMP stock solution in assay buffer. Before assaying, all samples and standards were acetylated. This was accomplished by placing 400 μl aliquots of each sample and standard in separate labelled tubes, adding 8 μl of triethylamine to each tube and in quick succession 7 μl of acetic anhydride, followed by vortexing. After acetylation the pH of the samples and standards was ≤ 5 .

Duplicate 100 μl aliquots of each acetylated standard and triplicate 100 μl aliquots of each acetylated sample were transferred to glass tubes. Diluted cGMP antiserum (25 μl) was added to each tube, followed by addition of 25 μl of ^{125}I -cGMP-TME. In addition to the standards, two tubes for estimation of non-specific binding and two tubes for estimation of total bound cGMP were included in the assay. The non-specific-binding tubes contained 100 μl of acetylated zero standard, 25 μl of γ -globulin buffer and 25 μl of ^{125}I -cGMP-TME. The total-bound tubes contained 100 μl of the acetylated zero standard, 25 μl of cGMP antiserum and 25 μl of ^{125}I -cGMP-TME. The assay tubes were covered and incubated overnight (about 20 h) at 4 °C.

The next day the assay was stopped by adding 50 μl of magnetic goat anti-rabbit secondary antibody to each tube, mixing and incubating all the tubes for 1 h at 4 °C. After incubation, 1 ml of ice-cold 12% PEG was added to each tube, and the tubes were vortexed before centrifugation for 20 min at 1300 g (4 °C). The supernatant was aspirated, and another 1 ml of 12% PEG added to each tube without mixing followed by centrifugation and aspiration of supernatant as before. The tubes were counted in a γ -radiation counter.

Ca^{2+} measurements

Intracellular Ca^{2+} measurements were performed using fura 2/AM as described previously [8]. Briefly, $[\text{Ca}^{2+}]_i$ in single cells in acinar configurations was measured by means of a digital-imaging system consisting of a Zeiss Axiovert 135 microscope equipped with a Zeiss Achrostat 40 $\times$ objective with a 1.3 numeric aperture, a CCD 72 video camera and a GenIIsys image intensifier from Dage-MTI (Michigan City, IN, U.S.A.) and MetaFluor software from Universal Imaging Corporation (West Chester, PA, U.S.A.). Cells were loaded with 2 μM fura 2/AM in RPMI 1640 medium (37 °C). After washing with Krebs–Ringer

solution, the cells were transferred to a chamber slide and placed on the microscope stage. Images were acquired by using a xenon lamp as the excitation light source, band-pass filters selecting for 340 and 380 nm (Optisk Laboratorium, Lyngby, Denmark) as well as a 510 nm cut-off filter for emission. $[\text{Ca}^{2+}]_i$ was measured according to the equation $[\text{Ca}^{2+}]_i = K_d \times (R - R_{\min}) / (R_{\max} - R) \times (S_{f2}/S_{b2})$, where the dissociation constant $K_d = 224$ nM, $S_{f2}/S_{b2} = 1.45$, $R_{\max} = 2.8$ and $R_{\min} = 0.1$. Agonists and agents were added from stock solutions to the cell chamber, giving a final concentration as indicated in the text. For investigations using the Mn^{2+} quench technique a DeltaRAM illuminator system from Photon Technology International, PhotoMed GmbH (Wedel, Germany) was applied.

We avoided the use of DMSO as solvent for any test agents in experiments involving SNAP stimulation, because DMSO in itself caused a drastic decrease in the SNAP-induced Ca^{2+} rise. The mechanism of this effect is not known to us, but might be due to nitrosylation of DMSO by SNAP-produced NO.

Intracellular release of cGMP from caged cGMP

Fura 2-loaded acini were suspended in a Krebs–Ringer buffer and placed on the microscope stage where the appropriate acini were focused on with the objective, followed by incubation for 2.5 min with 250 μM cell-permeable caged cGMP. The acinar configurations were then exposed to a 2 s UV flash from a 75 W xenon lamp with a long wavelength UG5 filter in the light path, after which $[\text{Ca}^{2+}]_i$ was measured as described above.

RESULTS

ADP-ribosyl cyclase activity in extracts from rat parotid acinar cells

ADP-ribosyl cyclase is the enzyme responsible for catalysing the conversion of $\beta\text{-NAD}^+$ to cADPR. A novel technique was used for determining the ADP-ribosyl cyclase activity in enzyme extracts from rat parotid acinar cells. The technique takes advantage of the fact that ADP-ribosyl cyclase is not very selective about the purine base of its substrate and therefore NGD^+ can be utilized as an alternative substrate to NAD^+ . The product of this reaction is a fluorescent analogue of cADPR, namely cGDP-ribose (cGDPR). Because neither the substrate, NGD^+ , nor the hydrolysis product, GDP-ribose, is fluorescent, it is possible to monitor the cyclization reaction directly by measuring the fluorescence development in an incubation mixture of NGD^+ with either purified enzyme or crude enzyme extracts [9].

Figure 1(a) shows traces from a representative experiment where different concentrations of NGD^+ were added to the cell extract. The rate of formation of the fluorescent compound was calculated by determining the slope of a straight line fitted to the initial increase in fluorescence emission (410 nm) with excitation at 300 nm. Figure 1(b) depicts the rate of initial fluorescence increase in the presence of different NGD^+ concentrations. The rate of fluorescence increase is shown as the percentage of $V_{\max}$, where $V_{\max}$ was obtained by fitting a mono-exponential curve to the data points. As seen in Figure 1(b) the reaction displayed saturation kinetics with an average apparent K_m value of 38 ± 12 μM NGD^+ ($n = 3$).

Since NAD^+ is the natural substrate of ADP-ribosyl cyclase, we tested whether NAD^+ could inhibit the cyclization of NGD^+ in a competitive manner. Experiments were performed with increasing NAD^+ concentrations in the incubation mixture of enzyme extract at fixed NGD^+ concentrations. We found that the NGD^+ cyclization reaction is competitively inhibited by NAD^+

Figure 1 Measuring ADP-ribosyl cyclase activity in extracts from rat parotid acinar cells

The cyclization reaction of NGD^+ to cGDPR was monitored by measuring the increase in fluorescence intensity after addition of NGD^+ at different concentrations. Excitation was at 300 nm and emission was at 410 nm. Representative traces are shown in (a). The rate of fluorescence increase was calculated by fitting a straight line to the initial fluorescence increase for each individual NGD^+ concentration (b). The data represent the means $\pm$ S.D. from three experiments. K_m 38 ± 12 μM NGD^+ ($n = 3$).

with a K_i value of 12 μM NAD^+ , consistent with our previous report [10]. Attempts to stimulate ADP-ribosyl cyclase activity with cGMP and G kinase were unsuccessful (results not shown). We also tested whether addition of apyrase to the enzyme extract, thus removing ATP, could lead to a decrease in the level of phosphorylation of ADP-ribosyl cyclase and hence a decrease in enzymic activity. A change in activity in the presence of apyrase was not observed. However, disruption of the integrated cellular processes caused by the ADP-ribosyl cyclase extraction procedure, where endogenous enzymes are diluted, might prevent such processes being discernible in this assay.

Fluorescence characteristics of cGDPR

To determine whether the fluorescent product of the cyclization reaction was cGDPR, the compound was isolated by HPLC and its fluorescence properties were characterized. For comparison cGDPR was synthesized by incubating NGD^+ with a soluble form of CD38, which is a lymphocyte antigen known to possess ADP-ribosyl cyclase activity [9]. Separating the CD38 incubation mixture by HPLC and using absorption spectrometry ($\lambda = 255$ nm) associated with the flow through the HPLC, we found that the eluate from the HPLC column could be separated into four peaks. The peaks corresponded to nicotinamide, leftover

Figure 2 Fluorescence characteristics of cGDPR

Corrected excitation and emission spectra were measured on the HPLC-isolated incubation product of NGD⁺ with CD38 (a) and parotid extract (b). The emission spectrum was measured at excitation 270 (solid line) and 300 nm (dashed line).

NGD⁺, cGDPR and GDP-ribose, which is the hydrolysis product of cGDPR. We then characterized the eluate fraction containing the cGDPR peak with respect to its excitation and emission spectra.

Figure 2(a) shows excitation and emission spectra obtained from the HPLC-isolated CD38-synthesized cGDPR. The excitation spectrum (emission at 400 nm) had two peaks at 245 and 270 nm and the emission spectrum (excitation at 270 nm) had a single peak at 385 nm. The emission peak remained the same irrespective of whether excitation was at 240, 270 or 300 nm (results not shown) and indicates that only one fluorescent compound was present, namely cGDPR.

Similarly, extracts from parotid acinar cells were pre-incubated with NGD⁺ as described above, and separated following the procedure for the CD38 incubation product. The product was injected into the HPLC column, and the fraction corresponding to cGDPR was used for characterization of excitation and emission spectra. Figure 2(b) shows the spectra for the parotid incubation product. The excitation spectrum is similar to the one in Figure 2(a), with the same two peaks at 245 and 270 nm. On the other hand the emission spectra (excitation at 270 and 300 nm) show a more complicated pattern. The emission spectrum (excitation at 270 nm) with a maximum at about 360 nm has a wide peak, indicating that the product contains more than one fluorescent compound. Excitation at 300 nm, however, resulted in an emission spectrum similar to the one observed for the CD38 product, consistent with the presence of cGDPR in the parotid incubation product.

Agonist-induced changes in [cGMP]

As mentioned above, cGMP has been suggested to regulate ADP-ribosyl cyclase activity. The [cGMP] in parotid acinar cells was measured by RIA (see the Materials and methods section). Figure 3 depicts the rise in [cGMP] in parotid acinar cells stimulated with 20 μ M isoproterenol, 40 μ M adrenaline or 1 μ M substance P. We found the unstimulated level of [cGMP] to be 1.68 ± 0.72 nM ($n = 15$) when expressed relative to total cell volume. The agonist-induced rise in [cGMP] had a rapid onset, with maximal concentrations observed after 5–15 s, and was found to be 150–300% of that seen in the unstimulated cells. Control experiments were performed in which suspensions of cells from the same preparation were stimulated with isoproterenol, adrenaline or substance P and $[Ca^{2+}]_i$ was measured. After adrenaline and substance P stimulation a rapid rise in $[Ca^{2+}]_i$ was observed, with a peak at about 5 s, followed by down-regulation to a plateau level. Isoproterenol stimulation, on the other hand, gave rise to no or only a slight transient rise in $[Ca^{2+}]_i$ (results not shown). When the cells were stimulated with 20 μ M of the α -adrenergic agonist, phenylephrine, no significant rise in [cGMP] was observed, although cells from the same preparation responded to phenylephrine stimulation with a rapid transient rise in $[Ca^{2+}]_i$ (results not shown).

The NO-induced rise in $[Ca^{2+}]_i$ is cGMP-mediated

We found that parotid acinar cells possess an enzyme that is able to convert NAD⁺ into cADPR and that receptor activation by

Figure 3 Agonist-induced changes in [cGMP]

The cellular [cGMP] was measured by RIA. The unstimulated values (at time 0) represent the means $\pm$ S.D. from three samples. The cells were stimulated as indicated with 20 μ M isoproterenol (ISO), 40 μ M adrenaline (Adr) or 1 μ M substance P (SP). Each curve is representative of three experiments.

Figure 4 The NO-induced rise in $[\text{Ca}^{2+}]_i$ and inhibition by ODQ

$[\text{Ca}^{2+}]_i$ was measured in single parotid acinar cells following the addition of $200 \mu\text{M}$ SNAP in the absence or presence of $10 \mu\text{M}$ ODQ (pre-incubated for 10 min). Traces represent averages of four experiments with 3–10 cells in each. Error bars represent S.E.M. * $P < 0.05$ (unpaired Student's t test).

Figure 5 cGMP mediates a rise in $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured in single parotid acinar cells, which were pre-incubated with $250 \mu\text{M}$ caged cGMP for 2.5 min, followed by photorelease of cGMP by a 2 s UV flash. The trace represents the average of eight cells from a representative experiment.

isoproterenol, adrenaline or substance P causes a rise in $[\text{cGMP}]$. These findings led us to examine whether changes in $[\text{cGMP}]$ could mediate changes in $[\text{Ca}^{2+}]_i$. NO, an activator of soluble guanylate cyclase, has previously been shown to cause an increase in the cellular $[\text{cGMP}]$ in parotid acinar cells [11,12]. We found that stimulation of rat parotid acinar cells with $200 \mu\text{M}$ SNAP, a well-characterized NO donor that releases NO spontaneously within seconds of being dissolved [13], induces a strong sustained rise in the $[\text{cGMP}]$ corresponding to an increase of 900% after 10 s [11]. To investigate whether this rise in $[\text{cGMP}]$ is associated with a change in $[\text{Ca}^{2+}]_i$, we measured $[\text{Ca}^{2+}]_i$ in individual fura 2-loaded cells from rat parotid acini. Figure 4 reveals that the $[\text{Ca}^{2+}]_i$ increased after SNAP addition to the bathing solution of the cells. The rise in $[\text{Ca}^{2+}]_i$ was most often saturated within 5–10 min and a down-regulation of $[\text{Ca}^{2+}]_i$ was only occasionally observed. The SNAP concentration applied

Figure 6 The cGMP-induced rise in $[\text{Ca}^{2+}]_i$ is mediated by a G kinase

$[\text{Ca}^{2+}]_i$ was measured in single parotid acinar cells. The cells were stimulated with $200 \mu\text{M}$ SNAP, followed by application of $100 \mu\text{M}$ Rp-8-pCPT-cGMPS, as indicated by the arrows. The figure shows the average trace of four cells from a representative experiment.

($200 \mu\text{M}$) was chosen since it gave rise to a reproducible increase in $[\text{Ca}^{2+}]_i$.

To investigate the process mediating the NO-induced Ca^{2+} release, the Ca^{2+} experiments were repeated in the presence of the inhibitor of soluble guanylate cyclases, ODQ. Following pre-incubation with $10 \mu\text{M}$ ODQ for 10 min the NO-induced rise in $[\text{Ca}^{2+}]_i$ was inhibited by about 50% measured at 90–260 s after stimulation with SNAP (Figure 4). The result indicates that the Ca^{2+} release was mediated by a soluble guanylate cyclase, synthesizing cGMP. This was further supported by experiments in which the fura 2-loaded cells were pre-incubated with cell-permeable caged cGMP for 2.5 min. $[\text{Ca}^{2+}]_i$ was monitored following a 2 s UV flash that photoreleased cGMP intracellularly (Figure 5). The results show that the released cGMP initiated a rise in $[\text{Ca}^{2+}]_i$ very similar to the one observed following NO addition, revealing that cGMP is the likely mediator of NO-induced Ca^{2+} release.

The NO/cGMP-induced rise in $[\text{Ca}^{2+}]_i$ is mediated by a G kinase

We investigated the possibility that the cGMP-induced increase in $[\text{Ca}^{2+}]_i$ was mediated by a pathway controlled by a G kinase. The effect of the specific, cell-permeable G-kinase inhibitor, Rp-8-pCPT-cGMPS, was tested under conditions where cells were first stimulated with SNAP, followed by application of the inhibitor (Figure 6). In order to ensure a constant NO concentration, the Rp-8-pCPT-cGMPS was added from a stock solution containing the same concentration of NO as the one in the bathing solution of the cells. The application of Rp-8-pCPT-cGMPS to the cells 100 s after SNAP stimulation resulted in an immediate decrease in $[\text{Ca}^{2+}]_i$, approaching the unstimulated level. The decrease in $[\text{Ca}^{2+}]_i$ observed after G-kinase inhibition reveals that a G kinase mediates the NO/cGMP-induced increase in $[\text{Ca}^{2+}]_i$.

NO induces release of Ca^{2+} from intracellular ryanodine-sensitive stores

To determine whether the NO/cGMP-induced rise in $[\text{Ca}^{2+}]_i$ was due to influx or release from intracellular stores, acini were stimulated with SNAP in the absence of extracellular Ca^{2+} . $[\text{Ca}^{2+}]_i$ was measured for 30 s, after which the cells were stimulated

Figure 7 The NO-induced rise in $[Ca^{2+}]_i$ is due to release of Ca^{2+} from intracellular stores

$[Ca^{2+}]_i$ was measured in single acinar cells from the rat parotid gland in Ca^{2+} -free Krebs-Ringer solution before and after stimulation with 200 μ M SNAP (a). The trace represents the average of eight cells from a representative experiment. On fura 2-loaded acinar cells the fluorescence intensity was measured at excitation wavelengths of 361.5 nm (the isosbestic point of fura 2) and 340 nm (b). 1 mM Mn^{2+} , 200 μ M SNAP, 20 μ M noradrenaline (NA) and 1 μ M ionomycin were added as indicated.

with SNAP (Figure 7a). This caused a rise in $[Ca^{2+}]_i$ of a magnitude similar to the one observed in the presence of extracellular Ca^{2+} , revealing that Ca^{2+} is released from intracellular stores following NO activation of cGMP synthesis. We investigated to what extent Ca^{2+} influx takes place during NO stimulation by using a Mn^{2+} -quenching technique. This technique takes advantage of the fact that at the isosbestic point (excitation at 361.5 nm in our imaging system) a change in $[Ca^{2+}]_i$ does not cause a change in fura 2 fluorescence. If Mn^{2+} influx takes place, mimicking a Ca^{2+} -influx component, Mn^{2+} will cause a quenching of fura 2 fluorescence. By changing between the excitation wavelengths of 340 nm and 361.5 nm using a monochromator, we found that the rise in $[Ca^{2+}]_i$ (excitation at 340 nm) induced by NO in the presence of 1 mM extracellular Mn^{2+} was not associated with a decrease in fura 2 fluorescence at excitation 361.5 nm (Figure 7b). Subsequent stimulation with noradrenaline did not lead to Ca^{2+} influx, whereas addition of the Ca^{2+} ionophore ionomycin resulted in an immediate decrease in fura 2 fluorescence. This finding reveals that NO/cGMP does not evoke a Ca^{2+} influx across the plasma membrane.

Figure 8 Ryanodine inhibits the NO-induced increase in $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in single parotid acinar cells before and after stimulation with 200 μ M SNAP in the presence or absence of 100 μ M ryanodine (pre-incubation for 15 min). The figure depicts the average $\Delta[Ca^{2+}]_i$ of five different experiments with 3–10 cells in each. Error bars represent S.E.M. * $P < 0.05$, ** $P < 0.01$ (unpaired Student's *t* test).

It is generally accepted that the RyR is involved in cADPR-induced Ca^{2+} release. We therefore tested whether the NO-mediated Ca^{2+} release was inhibited by ryanodine, a compound that blocks the RyR Ca^{2+} channel at concentrations of 100 μ M and above [14]. The fura 2-loaded cells were pre-incubated for 15 min with 100 μ M ryanodine, after which the cells were stimulated with 200 μ M SNAP. Figure 8 shows that in the presence of 100 μ M ryanodine, the NO-induced rise in $[Ca^{2+}]_i$ was inhibited by about 40%, measured 20–110 s after SNAP stimulation. The results reveal that ryanodine-sensitive Ca^{2+} stores are responsible for a considerable part of the cGMP-induced Ca^{2+} release.

DISCUSSION

Changes in [cGMP] induced by agonists

We investigated the possibility that Ca^{2+} -release processes can be controlled by a pathway in which agonists activate an endogenous soluble guanylate cyclase, leading to cGMP production from GTP. We found that stimulation of isolated rat parotid acini with either isoproterenol, adrenaline or substance P led to a rise in [cGMP] within seconds of stimulation. The peak was about 150–300% that of the unstimulated level. No significant rise in [cGMP] was observed when the α -adrenergic receptor was stimulated with phenylephrine, whereas $[Ca^{2+}]_i$ increased by about 100% under the same conditions. These findings are consistent with those of White and Klein [15], who showed that β -adrenergic stimulation of rat pinealocytes increases cGMP levels 2–4-fold, whereas α -adrenergic stimulation has little effect on cGMP accumulation [15]. However, when the pinealocyte α - and β -adrenergic receptors are stimulated simultaneously, cGMP accumulation is increased > 100-fold. The relatively small rise in [cGMP] observed after stimulation of parotid acini with adrenaline, which activates both α - and β -adrenergic receptors, suggests that this kind of potentiation is not found in rat parotid acinar cells. It could easily be conceived that adrenaline activates synthesis of cGMP through receptor-mediated NO production [11]. However, with respect to substance P, a much smaller NO synthesis is observed [11] that might, in principle, be sufficient for activating the guanylate cyclase. It is also a possibility that

substance P activates guanylate cyclase via a pathway that is not strongly dependent on NO synthesis.

NO is a likely physiological activator for soluble guanylate cyclases in parotid acinar cells, since previous reports have shown that NO synthase, the enzyme that catalyses NO synthesis, is localized in the nerve endings surrounding acini in parotid glands [1]. It is therefore conceivable that an essential step in the stimulus–secretion coupling is that NO is synthesized following a Ca^{2+} rise in the neuron, and diffuses into the acinar cell simultaneously with activation of receptors by neurotransmitters released from neuronal vesicles.

We have reported previously that SNAP stimulation of parotid acinar cells leads to a powerful rise in [cGMP] that, when applying 200 μM SNAP, is many-fold larger than that observed in this study following receptor stimulation. Thus the data suggest that, under physiological conditions, NO released from nerve endings is capable of inducing much larger rises in [cGMP] than the agonist-induced pathways. This may reflect that exogenous NO stimulates guanylate cyclases widely throughout the cell, whereas the agonist-stimulated pathway may only stimulate cGMP synthesis at particular locations in the cell. Since [cGMP] was measured on whole cells, a large ‘local’ increase would be masked.

An increase in [cGMP] induces a G-kinase-mediated release of Ca^{2+} from intracellular stores

Our results show that an essential role of the increases in cellular [cGMP] is to regulate the $[\text{Ca}^{2+}]_i$. This is evident from our finding that stimulation by SNAP induced a continuous increase in $[\text{Ca}^{2+}]_i$. The NO-induced rise in $[\text{Ca}^{2+}]_i$ in parotid acinar cells was mediated by cGMP to a large extent, as shown by the ability of ODQ to diminish the release of Ca^{2+} by about 50%. ODQ is a selective inhibitor of soluble guanylate cyclases and NO-induced cGMP synthesis is reduced by about 75% in the presence of ODQ [16]. To exclude the possibility that the observed Ca^{2+} rise was due to nitrosylation, as reported for skeletal and cardiac RyRs [17], acini were challenged with an increase in [cGMP], photoreleased from caged cGMP. The result was an increase in $[\text{Ca}^{2+}]_i$ very similar to the one observed after NO stimulation and therefore confirmed that cGMP plays a role in the NO-induced Ca^{2+} -release pathway in rat parotid acinar cells. We chose to use photorelease of caged cGMP to increase the cellular [cGMP] in these experiments, to mimic the very fast increase in [cGMP] observed after receptor and NO stimulation. The ability of Rp-8-pCPT-cGMPS to diminish the NO/cGMP-induced rise in $[\text{Ca}^{2+}]_i$ immediately and potently reveals that the NO/cGMP-induced Ca^{2+} release is mediated by a G kinase in rat parotid acinar cells.

We found two lines of evidence for Ca^{2+} being solely recruited from intracellular stores. First, in the absence of external Ca^{2+} , NO is able to induce a rise in $[\text{Ca}^{2+}]_i$ of the same magnitude as the rise observed in the presence of external Ca^{2+} . Secondly, Mn^{2+} -quenching studies revealed that a Ca^{2+} -influx component is not induced by NO stimulation of parotid acinar cells. Thus our experiments show that the NO-induced rise in $[\text{Ca}^{2+}]_i$ is caused by Ca^{2+} released from intracellular stores.

ADP-ribosyl cyclase activity in rat parotid acinar cells

In this study the presence of enzymes with ADP-ribosyl cyclase activity was identified in extracts from rat parotid acinar cells. We characterized the enzymic activity as well as the product formed during incubation of the enzyme with NGD^+ . One product was cGDPR, a fluorescent analogue of cADPR, and the

synthesis of cGDPR was found to be competitively inhibited by the natural substrate of the enzyme, NAD^+ . Since both NGD^+ and NAD^+ compete for the same substrate site, one may assume that the measured K_i value for NAD^+ inhibition of the NGD^+ cyclization reaction is roughly equal to the K_m value of NAD^+ binding to the substrate site. Thus the ADP-ribosyl cyclase in parotid acinar cells is able to bind NAD^+ and convert it into cADPR in the physiological intracellular NAD^+ concentration range, 20–50 μM [18].

In addition, we provide evidence for there being more than one product of the cyclization reaction of NGD^+ . This notion is made plausible by reports of Genazzani and Galione [19], in which they describe that ADP-ribosyl cyclase is capable of catalysing the synthesis of not only one, but various compounds from different substrates. Using $\beta\text{-NAD}^+$, $\beta\text{-NADP}^+$ and nicotinic acid as substrates the enzyme can synthesize nicotinic acid–adenine dinucleotide (NAAD), nicotinic acid–adenine dinucleotide phosphate (NAADP) and 2'-P-cADPR as well as cADPR. De Flora et al. [20] reported that ADP-ribosyl cyclase catalysed synthesis of a dimeric ADP-ribose, (ADP-ribose)₂, which does not itself release Ca^{2+} from sea urchin egg microsomes, but specifically potentiates the Ca^{2+} -releasing activity of sub-threshold concentrations of cADPR.

ADP-ribosyl cyclases have now been identified in a wide variety of mammalian tissues including liver, brain, heart, spleen and kidney [18], as well as in sea urchin eggs where they were first discovered [4]. The finding of enzymes with ADP-ribosyl cyclase activity in acini from exocrine glands points to the possibility that it is a ubiquitous enzyme with important implications for the Ca^{2+} homeostasis of cells.

Ca^{2+} -release processes involving cADPR and the RyR

As mentioned above, cADPR is most probably involved in Ca^{2+} mobilization from intracellular stores through the RyR and is thus part of Ca^{2+} -induced Ca^{2+} -release processes [21]. We found that the NO-induced Ca^{2+} release in parotid acini was inhibited by about 40% in the presence of ryanodine, consistent with a large part of the Ca^{2+} release being mediated by a mechanism responsible for Ca^{2+} -induced Ca^{2+} -release processes. The incomplete inhibition in the presence of ryanodine is due to the activation by cGMP of $\text{Ins}(1,4,5)\text{P}_3$ production, leading to Ca^{2+} release from ryanodine-insensitive stores [11].

Synthesis of cADPR presents the crucial step in the model for G-kinase-mediated Ca^{2+} -release processes activated by NO/cGMP in sea urchin eggs [3]. In this study we have shown that the G-kinase-mediated NO/cGMP-activation of Ca^{2+} release was inhibited by ryanodine. Thus the presence of ADP-ribosyl cyclase activity in parotid acinar cells as well as the ability of cADPR to release Ca^{2+} from ryanodine-sensitive stores in these cells [6,22] points to the possibility that a similar pathway to the one described for sea urchin eggs exists in parotid acinar cells, where a G kinase is responsible for increasing the Ca^{2+} permeability of the RyR via synthesis of cADPR by ADP-ribosyl cyclase.

A model for cGMP-mediated Ca^{2+} release in rat parotid acinar cells

In summary, we here report that the [cGMP] increases in parotid acinar cells in response to isoproterenol, adrenaline and substance P stimulation. We also show that the NO-induced rise in [cGMP] causes a G-kinase-mediated release of Ca^{2+} from intracellular ryanodine-sensitive stores. In addition, we provide evidence for the presence of ADP-ribosyl cyclase activity in rat parotid acinar cells. The results presented in this study lead us to propose the

Figure 9 Model for a cGMP/cADPR-mediated Ca^{2+} -release process in rat parotid acinar cells

The figure shows the key steps leading from receptor or NO activation, to formation of cADPR and Ca^{2+} release. Some essential tools for elucidating the role of each step in this pathway are the inhibitors of the soluble guanylate cyclase (ODQ), the G kinase (the cGMP analogue Rp-8-pCPT-cGMPS) and the RyR (ryanodine). Their points of interaction are indicated by zig-zag arrows in the figure. For further details see text. SP, substance P; Adr, adrenaline; ISO, isoproterenol; PIP_2 , PtdInsP_2 ; IP_3 , InsP_3 ; PLC, phospholipase C; IP_3R , InsP_3 receptor.

model presented in Figure 9 for cGMP-mediated Ca^{2+} release in rat parotid acinar cells. According to this model, NO as well as β -adrenergic and neurokinin (substance P) receptor stimulation leads to the activation of a soluble guanylate cyclase. The formed cGMP then activates a G kinase, which phosphorylates and activates an ADP-ribosyl cyclase. The activated ADP-ribosyl cyclase then converts β - NAD^+ to cADPR, which in turn interacts with the RyR. The Ca^{2+} sensitivity of the Ca^{2+} -release complex is thereby increased, leading to release of Ca^{2+} from intracellular stores. Although the results presented in this study allow the possibility of such a pathway, conclusive evidence is necessary to establish the link between the G kinase and ADP-ribosyl cyclase. The model also shows that cGMP synthesis leads to phospholipase C-mediated production of $\text{Ins}(1,4,5)\text{P}_3$ [11], a process that is presumably mediated by a G kinase, as illustrated in Figure 9 by the dashed arrow. This pathway accounts for the Ca^{2+} release that is not inhibited by ryanodine.

It is well established that in acinar cells the initial rise in $[\text{Ca}^{2+}]_i$ evoked by acetylcholine, noradrenaline and substance P is due to a rapid rise in the $\text{Ins}(1,4,5)\text{P}_3$ concentration [7,23]. However, cellular control of $[\text{Ca}^{2+}]_i$ also depends on $\text{Ins}(1,4,5)\text{P}_3$ -insensitive Ca^{2+} stores such as Ca^{2+} -induced Ca^{2+} -release processes via the RyR channel. According to our model, one of the key factors in controlling the Ca^{2+} permeability of the RyR channel is the enzyme ADP-ribosyl cyclase.

In 1996, Lee [24] proposed two different models in which cADPR might exert its effect, either as a messenger or as a modulator. In the messenger model cADPR is synthesized in response to an external stimulus, e.g. receptor stimulation, and leads to release of Ca^{2+} from intracellular stores in a manner similar to that of $\text{Ins}(1,4,5)\text{P}_3$. As a modulator cADPR would function by being synthesized at a constant level sufficient to sensitize the RyR to small increases in $[\text{Ca}^{2+}]_i$. At present, we favour the idea that cADPR operates somewhere in between these two extremes in parotid acinar cells. Since our data show that even the largest fluctuations in [cGMP] caused by SNAP

stimulation result in relatively minor changes in $[\text{Ca}^{2+}]_i$, we hypothesize that a change in [cGMP] results in a modulation of the Ca^{2+} permeability, although being unable to evoke a major change in $[\text{Ca}^{2+}]_i$ by itself, as seen in the case of isoproterenol stimulation. Thus the role of cADPR synthesized in response to G-kinase activation of ADP-ribosyl cyclase (messenger role) might be to modulate the RyR channel permeability by increasing the RyR sensitivity to Ca^{2+} . We propose that cADPR, via the cGMP/G kinase pathway, forms the basis for prolonged Ca^{2+} -induced Ca^{2+} release in the acinar cells, but does not contribute to the rapid Ca^{2+} transients observed upon receptor activation.

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