Calcium-dependent release of NO from intracellular S-nitrosothiols

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The paper describes a novel cellular mechanism for rapid calcium-dependent nitric oxide (NO) release. This release occurs due to NO liberation from S-nitrosothiols. We have analysed the changes of NO concentration in acutely isolated pancreatic acinar cells. Supramaximal acetylcholine (ACh) stimulation induced a Ca^{2+} -dependent increase in the fluorescence in the majority of cells loaded with the NO probe DAF-FM via a patch pipette. The ACh-induced NO signals were insensitive to inhibitors of calmodulin and protein kinase C but were inhibited by calpain antagonists. The initial part of the NO signals induced by $10\text{ }\mu\text{M}$ ACh showed little sensitivity to inhibition of NO synthase (NOS); however, cell pretreatment with NO donors (increasing cellular S-nitrosothiol contents) substantially enhanced the initial component of NO responses. Pancreatic acinar cells were able to generate fast calcium-dependent NO responses when stimulated with physiological or supramaximal doses of secretagogues. Importantly, the source of this NO is the already available S-nitrosothiol store rather than *de novo* synthesis by NOS. A similar mechanism of NO release was found in dorsal root ganglia neurons.

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Introduction

Nitric oxide (NO) is a messenger molecule that is involved in many signalling processes, via the cellular cyclic guanosine-3',5'-monophosphate (cGMP) pathway and through the nitrosylation of regulatory thiols. In the exocrine pancreas, exogenous NO, its precursor L-arginine or the cGMP analogue 8-Br-cGMP has been shown to modulate amylase secretion, most probably by upregulating pancreatic blood flow and/or by affecting neurotransmitter release from neuronal terminals. Endogenous NO has been known to regulate the balance between proliferation and apoptosis in the pancreas (Trulsson *et al*, 2002) and protect cells during pancreatitis

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(Molero *et al*, 1995; Werner *et al*, 1998; Andrzejewska *et al*, 2002).

Pancreatic NO synthase (NOS) protein expression is primarily extra-acinar (Yago *et al*, 2001; DiMagno *et al*, 2004). There is a controversy regarding the presence of constitutive NOS isoforms (and, consequently, the ability to synthesize NO) in the most abundant cells in the pancreas—the acinar cells. Some investigations show absence of constitutive NOS in pancreatic acini (Worl *et al*, 1994; Umehara *et al*, 1997; Al Mufti *et al*, 1998; Ember *et al*, 2000), whereas others report detectable levels of NOS mRNA (Jaworek *et al*, 2000), protein (Xu *et al*, 1997; Nam *et al*, 1998; DiMagno *et al*, 2004) or NOS activity (Wrenn *et al*, 1994; Molero *et al*, 1995; Xu *et al*, 1997; Ahn *et al*, 1998; Jaworek *et al*, 2000). There are also contradictions in the reports about the effects of calcium mobilization (which is essential for activating constitutive NOS isoforms via Ca-calmodulin) on NO production and downstream components of the NO signalling pathway in the isolated acinar cells/acini. Little or no change in cGMP production during agonist stimulation was described (Gilon *et al*, 1995; Yoshida *et al*, 1997). In contrast, other studies have reported a substantial increase in [cGMP]_c upon stimulation of cells with calcium mobilizing secretagogues (Gardner and Rottman, 1980; Pandol and Schoeffield-Payne, 1990; Gukovskaya and Pandol, 1995; Ahn *et al*, 1998).

The aim of this study was to employ fluorescent imaging techniques, using NO-sensitive dyes, for the investigation of NO production in pancreatic acinar cells. The results of this study were unexpected—we found that the fast initial component of the $[\text{NO}]_i$ increase in stimulated acinar cells occurred owing to Ca^{2+} -dependent NO release from intracellular heavy molecular weight S-nitrosothiols. This may be an important general mechanism of redistribution and release of NO.

Results

Testing different loading protocols for fluorescent NO probes

In order to optimize the NO measurement, we tested different protocols of cell loading with NO-sensitive dyes. We used two fluorescent NO-sensitive probes DAF-2 DA and DAF-FM. The initial experiments were conducted using the membrane-permeable dye DAF-2 DA, which undergoes de-esterification to DAF-2 inside the cells. The experiments were performed on isolated cells or small clusters of usually 2–3 cells (Figure 1). The amplitude of fluorescence changes in response to $500\text{ }\mu\text{M}$ of spermine–NO complex (sp/NO) in these experiments was small (blue traces in Figure 1A and C, $n = 13$). It is important to note that the existing NO-sensitive fluorophores react with products of NO oxidation by molecular oxygen rather than NO itself (Kojima *et al*, 1999; Espey *et al*, 2001). These reactive nitrogen species (RNS), unlike the NO radical, are able to react with a number of cell constituents including ascorbate (Espey *et al*, 2001), sulphhydryl groups of proteins

Figure 1 Effects of the NO donor sp/NO 500 μ M on the fluorescence of DAF-2 and DAF-FM introduced into the cells by different loading protocols. The representative normalized traces of fluorescence changes in cells loaded with DAF-2 DA and DAF-FM (A), their images (B) and average traces of fluorescence (C). The traces on these and other figures were normalized to compensate for the flux of the indicator from the pipette, leakage and bleaching. (A) Normalized fluorescent signals recorded from intact (blue trace, image Ba) and patched (red trace, image Bb) cells loaded with DAF-2 DA and patched cells loaded with DAF-FM (black trace, image Bc). (B) Transmitted light and fluorescence images of corresponding cells. Regions of interest from which fluorescence was recorded are shown by dotted lines. Bars correspond to 10 μ m. (C) Average traces of normalized fluorescence with s.e.m. error bars. Blue corresponds to intact DAF-2 DA-loaded cells ($n = 13$). Red shows responses of the stained cells, in which the cytosol was dialysed with patch pipette ($n = 4$). Green represents cells pre-treated with *N*-ethylmaleimide to deplete -SH groups ($n = 8$). A substantial proportion of NEM-treated cells died towards the end of experiments; we therefore show only first 900 s of the trace when all cells are still alive. Black shows responses of cells loaded with DAF-FM via the patch pipette ($n = 4$).

and glutathione (Espey *et al*, 2001; Hess *et al*, 2005). These reactions are known to compromise the sensitivity of NO detection by NO-sensitive dyes, especially if the dye concentration falls to substantially less than 1 mM (Rodríguez *et al*, 2005). It is therefore expected that perfusion of the cytosol, in the whole-cell patch clamp mode, and removal of low molecular weight nitrosation scavengers should result in an increase in the sensitivity of NO detection. Indeed, the amplitude of the fluorescence changes was larger in cells that, following DAF-2 DA loading, were dialysed via a patch pipette (compare blue and red traces in Figure 1A and C) ($n = 4$). In these experiments, a substantial proportion of DAF-2 was lost from the cytoplasm but some indicator remained, presumably owing to compartmentalization or binding to cell constituents. To verify that endogenous reduced thiols are responsible for the observed decrease in

efficiency of NO detection in intact (not patched) cells, we chemically depleted the -SH pool in the cells. This was achieved by incubating cells with 200 μ M *N*-ethylmaleimide for 10 min. The responses to sp/NO in these cells were significantly enlarged compared to responses recorded in intact cells (Figure 1C, green triangles, $n = 8$), but were not significantly different from those detected in the cells dialysed via the patch pipette. These results indicate that in pancreatic acinar cells, endogenous mobile reduced thiols do decrease the sensitivity of NO detection by DAF indicators.

Our attempts to use another fluorescent NO probe DAF-FM DA (membrane-permeable form) for loading pancreatic acinar cells were unsuccessful. A substantial proportion of cells died or was damaged. However, we found that DAF-FM, when loaded via the patch pipette, is very sensitive to sp/NO (Figure 1A–C, $n = 4$) and well tolerated by the cells. In our further experiments, we used both DAF-2 and DAF-FM; however, DAF-FM was our main probe because of its high sensitivity (Figure 1) and reported lower pH dependence (Kojima *et al*, 1999).

NO production by supramaximal muscarinic receptor stimulation

We next investigated whether stimulation of pancreatic acinar cells by a supramaximal concentration of the calcium-releasing secretagogue acetylcholine (ACh) can trigger NO release. The initial experiments were conducted on cells loaded with DAF-2 DA, and ACh was found to increase the cell fluorescence (Figure 2A and B), but as with application of sp/NO (see blue versus red traces in Figure 1), the changes of fluorescence were clearer in cells subjected to intracellular dialysis via a patch pipette (blue versus red traces in Figure 2E). ACh at 10 μ M produces significant (> 3 s.d.) changes of fluorescence in 28% (five out of 18) of the intact cells (data from a responding cell is shown in Figure 2A) compared to 67% (six out of eight) patched cells (example shown in Figure 2B). A slightly higher percentage of responses to ACh (79% = 30 out of 38) was seen when cells were loaded with DAF-FM via the patch pipette (see Figure 2C, averaged response is shown as a black trace in Figure 2E). The whole-cell patch clamp mode also enabled us to measure calcium-dependent current, which can serve as an indicator of the $[Ca^{2+}]_c$ rise in this cell type (Petersen, 1992). ACh stimulation triggered a Ca²⁺-dependent Cl[−] current, which developed faster than changes in DAF-FM fluorescence (the calculated time between the peak of the current and the peak of the first derivative of the normalized fluorescence (dF_{norm}/dt) was 123 ± 31 s). In some cases, after 5–10 min of loading with DAF-FM, the patch pipette was withdrawn and the cell was allowed to reseal before the stimulation. In five out of 14 cells (36%), ACh produced resolvable responses (Figure 2D). The reduced proportion of responding cells could be explained by the traumatic effect of pipette withdrawal. Importantly, our ability to detect clear DAF-FM responses at least in some of these cells suggests that the fluorescence changes reflect NO responses and not just the redistribution of the indicator between the cytosol and the patch pipette.

BAPTA inhibits ACh-induced responses of DAF-FM

Strong buffering of the intracellular Ca²⁺ concentration (replacing 0.2 mM EGTA by 10 mM BAPTA plus 2 mM Ca)

Figure 2 NO production induced by 10 μ M ACh. (A) Normalized trace of fluorescence for an intact cell loaded with DAF-2 DA. Only five out of 18 tested cells showed resolvable increase in fluorescence upon ACh stimulation. (B) Normalized trace of fluorescence (red) and current recorded at -30 mV holding potential (purple) for a cell loaded with DAF-2 DA and then subjected to wash out of the cytosolic dye by intracellular perfusion with the dye-free patch pipette solution. (C) Normalized trace of fluorescence (black) and current (purple) for a cell loaded with DAF-FM through the patch pipette. (D) Normalized trace of fluorescence for a cell loaded with DAF-FM through the patch pipette and stimulated with ACh following pipette withdrawal. The pipette was withdrawn following 10 min of intracellular perfusion in whole-cell patch clamp configuration and the fluorescence recording started approximately 1 min after the withdrawal of the pipette. (E) Average normalized traces of fluorescence with corresponding s.e.m. Blue trace represents fluorescence in intact cells loaded with DAF-2 DA ($n = 18$, calculated for all cells including the majority of cells that did not show fluorescence changes upon ACh application). Red trace represents average response in cells loaded with DAF-2 DA and subsequently dialysed by patch pipette ($n = 8$). Black trace shows normalized fluorescence changes in cells loaded with DAF-FM via patch pipette ($n = 38$).

eliminated the ACh-induced, Ca²⁺-dependent Cl⁻ currents in nine out of 12 cells, whereas in the remaining cells much smaller calcium responses were recorded compared to the cells dialysed with the weak Ca²⁺ buffer. Such tight Ca²⁺ buffering dramatically inhibited the ACh (10 μ M)-induced fluorescence changes (Figure 3A), indicating that the [Ca²⁺]_c rise is essential for the ACh-stimulated NO production.

Reduced glutathione and NO scavengers suppress ACh-induced [NO]_i rise

The responses of DAF-FM to supramaximal cholinergic stimulation were significantly inhibited when 25 mM reduced glutathione (GSH)—an endogenous cytosolic thiol known to react with one electron oxidized or reduced products of NO (nitrosonium and nitroxyl)—was added to the patch pipette solution (Figure 3A, blue trace, $n = 14$). An exogenous NO

Figure 3 Pharmacological inhibition and potentiation of ACh-stimulated NO signals: negative control experiments, effect of BAPTA loading, NOS inhibition and the role of intracellular S-nitrosothiols. (A) Effect of strong [Ca²⁺]_c chelation by 10 mM BAPTA plus 2 mM Ca²⁺ in the patch pipette solution (brown trace, $n = 12$). Here and in other parts of this figure and in Figure 6, the black trace shows control NO responses in conditions of mild [Ca²⁺]_c buffering by 0.2 mM EGTA (same as in Figure 2E). The blue trace on this part of the figure shows the effect of addition of GSH (25 mM) to the patch pipette solution ($n = 14$). The asterisks by the time axis indicate that the data of the correspondingly coloured trace at the given time points are significantly different ($P < 0.05$) from those of control (same applies to parts B and C). (B) Effect of NO scavenger Fe-DTCS complex added to the patch pipette solution is represented by green trace ($n = 14$). Violet trace shows results of inclusion of superoxide dismutase to the patch pipette solution plus superoxide scavenger TEMPOL (both in intra- and extracellular solution) ($n = 11$). Red trace shows results of experiments in which DAF-FM was substituted by inactive NO probe, which was obtained by pretreatment of DAF-FM with excess of NO before loading into the patch pipette ($n = 11$). (C) Effect of NOS inhibition by L-NAME and 7-NI is represented by pink trace ($n = 19$). The effect of cell treatment with NO donor in the presence of blockers of NOS is shown by cyan trace ($n = 23$). The yellow trace depicts results of the β -escin perforated patch experiments ($n = 9$). This patch clamp configuration should preserve the protein composition of the cytosol. In these experiments, the pipette solution was also supplemented with substrate (L-arginine) and cofactors (FAD, FMN, BH₄ and NADPH) for NO synthesis.

scavenger Fe²⁺-DTCS (DTCS, dithiocarbonylsarcosine) (10 μ M), when added to the patch pipette, effectively suppressed the ACh-induced NO increases (Figure 3B, green trace, $n = 14$). Other structurally unrelated NO scavengers,

haemoglobin (10 mg/ml, $n=6$; Supplementary Figure S1A) and C-PTIO (30 μ M, $n=4$, not shown), also effectively inhibited ACh-induced NO responses, confirming the specificity of NO measurements with DAF-FM. Finally, an inactive probe, DAF-FM T, which was produced by prior saturation of DAF-FM with nitrosating species, was essentially unresponsive when loaded into the cell via the patch pipette (Figure 3B, red trace, $n=11$). It is worth noting that in some negative control experiments, the supramaximal ACh stimulation still caused a small increase in fluorescence (less than 10% in average compared with approximately 40% in untreated cells). Electrodiffusion of DAF-FM between the patch pipette and the patched cell or between the patched cell and the adjacent cells in the cluster could be the possible explanation for this small artefact (see Mathias *et al*, 1990). We next checked directly the contribution of electrodiffusion effects in the ACh-induced change of cell fluorescence.

A group of cells was stimulated with ACh 10 μ M under conditions that minimize these two electrodiffusion effects. Cells were incubated in 1-octanol at 0.5 or 1 mM concentrations, which blocks gap junctions in these cells (Stauffer *et al*, 1993; Deutsch *et al*, 1995), and held at +5 mV holding potential instead of -30 mV in other groups ($n=14$) to reverse and diminish chloride current (I_{Cl^-}). The averaged ACh-induced fluorescence changes in these cells were slightly smaller than in control group (e.g. by 10% for the last time points of recordings) but the difference was not statistically significant ($n=14$, not shown). This result suggests that the fluorescence responses to calcium-releasing agonists reflect genuine changes in $[NO]_i$ rather than the electrodiffusion artefacts.

As the superoxide anion has been shown to produce an extra increase in fluorescence of DAF indicators in the presence of NO donors (Jourdain *et al*, 2002), we checked whether the responses to ACh can be explained by superoxide. In these experiments, the specific superoxide scavengers TEMPOL 1 mM and superoxide dismutase 3700 U/ml were added in the patch pipette solution, while the membrane-permeable TEMPOL was also present in the extracellular solution. These modifications failed to inhibit the ACh-induced changes in fluorescence (Figure 3B, violet trace, $n=11$), ruling out superoxide as the substance underlying the observed DAF-FM responses.

Secondary role of NOS in the initial component of ACh-induced NO release

To check the role of NOS in the ACh-induced NO release, we incubated cells with the constitutive NOS antagonists L-NAME (100 μ M) and 100 μ M of 7-nitroindazole (7-NI). The inhibitors were added to the extracellular solution at least 30 min before the experiments and were present in extra- and intracellular solutions throughout the experiments. This inhibition of NOS effectively suppressed the late component (after approximately 600 s; compare purple and black traces in Figure 3C), suggesting some delayed NOS contribution to the ACh-induced NO responses, but the initial component of the response (first few hundred seconds) was unaffected ($n=19$). It is plausible that the decline of cell fluorescence, which occurs in the presence of NOS blockers after 1000 s (Figure 3C, purple trace), is owing to diffusion of the NO-bound form of the indicator (DAF-FM T) into the patch

pipette at such a rate that the loss of DAF-FM T exceeds its production.

The results from the fluorescence experiments were confirmed by using a NO-sensitive electrode in a suspension of isolated pancreatic acinar cells (approximately 25×10^6 cells in 5 ml total volume). In six out of 10 preparations, ACh (10 μ M) induced an increase in NO concentration in the presence of L-NAME and 7-NI (Supplementary Figure S1B), and these responses were similar to those observed when L-NAME and 7-NI were omitted.

We also tried to facilitate NOS activity. In these experiments, L-arginine and the NOS cofactors FAD, FMN, BH₄ and NADPH were added to the patch pipette solution. In these experiments, we used the β -escin perforated patch clamp configuration (Fan and Palade, 1998), which should largely preserve the protein composition of the cells, while allowing easy access of small molecules to the cytosol. Such modifications did not increase the ACh-induced NO production ($n=12$, compare orange and black traces in Figure 3C), showing that 'whole-cell' dialysis conditions caused no adverse effects on NOS. These findings altogether imply little importance of NOS in the initial, fast component of the ACh-induced NO release.

Evidence for the role of intracellular S-nitrosothiols in ACh-induced NO release

The intracellular bound NO pool, which includes S-nitrosothiols, heme-NO products, dinitrosyl iron complexes and N-nitrosamines, is a possible source for NO liberated by ACh stimulation. We therefore checked if increasing the intracellular S-nitrosothiol content would affect the ACh-induced NO responses. In these experiments, cells were preincubated for 30 min with the NO donor sp/NO 100 or 200 μ M (which was removed just before patching). Such a treatment raised the amount of intracellular S-nitrosothiols (see next paragraph) and substantially increased the magnitude of the NO signals induced by supramaximal ACh stimulation ($n=23$, compare cyan trace and black trace in Figure 3C). Notably, these experiments were conducted in the presence of the NOS blockers L-NAME and 7-NI. This result suggests that the amplitude of the ACh-induced NO signal correlates with the size of the intracellular S-nitrosothiol pool.

To investigate whether heavy molecular weight S-nitrosothiols were present inside the dialysed cells in a sufficient amount to account for the ACh-induced NO release, we exposed pancreatic acinar cells to HgCl₂ (10 mM) supplied with ionomycin (10 μ M). Hg²⁺ is known to catalyse S-NO bond cleavage (Saville, 1958) and has been used for quantification of S-nitrosothiols. The treatment gave rise to a strong (more than five-fold) increase in fluorescence in the cells loaded with DAF-FM (Figure 4A). At variance with the reported interaction of Hg²⁺ with DAF-2 dye (Rodriguez *et al*, 2005), HgCl₂ had no effect on the fluorescence of DAF-FM (Figure 4B, solid line). However, HgCl₂ added to the mixture of DAF-FM and S-nitrosothiol glyco-SNAP (II) (which by itself is resistant to spontaneous decomposition) produced a strong fluorescence rise (Figure 4B). The amount of intracellular S-nitrosothiols sensitive to Hg²⁺ increased after pretreatment of the cells with NO donors (Figure 4, inset). The amplitude of typical DAF-FM fluorescence responses to supramaximal (10 μ M) ACh stimulation was less than 10% of that caused by HgCl₂ (Figures 3 and 4A).

Figure 4 Comparison of ACh-induced NO signal and NO change induced by Hg²⁺ (Saville reaction of Hg²⁺ with intracellular S-nitrosothiols). (A) Representative normalized trace of fluorescence for a cell loaded with DAF-FM through the patch pipette and sequentially stimulated by ACh and Hg²⁺ in the presence of ionomycin ($n=9$). (Inset) Effect of cell incubation with the NO donor sp/NO 100 μM (b), 200 μM (c), 500 μM (d), with NOS blockers L-NAME and 7-NI (e) and sp/NO 100 μM in the presence of NOS blockers (f) on the concentration of intracellular S-nitrosothiols accessed by Saville reaction. The results are represented as fold increase against control (a). (B) Solid line indicates that Hg²⁺ has no effect on DAF-FM fluorescence in the absence of NO. The arrows (under the trace) show timing of Hg²⁺ addition to the cuvette. Dashed line represents cell-free measurement of NO cleavage from a S-nitrosothiol (glyco-SNAP (II)) by Hg²⁺ ions. Arrows (above the trace) show the timing of Glyco-SNAP(II) and Hg²⁺ addition.

NO production caused by agonist- or non-agonist-induced [Ca²⁺]_i elevation

Similar to 10 μM ACh, supramaximal stimulation with cholecystokinin (CCK, 10 nM) elicited a [NO]_i increase revealed by DAF-FM loaded via the patch pipette (Figure 5A, three out of four cells). The physiological concentrations of CCK (5–10 pM) and a low concentration of ACh (50 nM) were also able to evoke measurable NO production (Figure 5B and C) in four out of 12 and two out of six cells, respectively. We found that thapsigargin (Tg), an inhibitor of the ER Ca-ATPase, caused a detectable [NO]_i rise in 11 out of 14 cells. These experiments show that a secretagogue-independent [Ca²⁺]_i increase is sufficient to trigger a NO response. It is important to note that a [NO]_i increase could be produced by both Tg-induced release of calcium from the ER and by Ca²⁺ influx through the plasma membrane (Figure 5D). Thus, Ca-releasing secretagogues can induce a measurable increase in the NO level, when acting in supramaximal and physiological concentrations and a [Ca²⁺]_i rise itself is sufficient to trigger the NO release.

Figure 5 NO production induced by different doses of calcium-releasing secretagogues and Tg. (A) Effect of a high dose of CCK on NO production (normalized DAF-FM fluorescence, black squares) and on Ca²⁺-dependent Cl⁻ current (solid black line) ($n=4$). (B) Oscillations of Ca²⁺-dependent Cl⁻ current (black line) and NO production (normalized DAF-FM fluorescence, black squares) induced by a low 'physiological' concentration of CCK ($n=12$). (C) Ca²⁺-dependent Cl⁻ current (black line) and NO production (normalized DAF-FM fluorescence, black squares) induced by a low concentration of ACh ($n=6$). (D) Ca²⁺-dependent Cl⁻ current (black line) and NO production (normalized DAF-FM fluorescence, black squares) induced by Tg initially added in extracellular Ca²⁺-free medium with subsequent calcium re-addition ($n=14$).

The NO responses are independent of calmodulin, protein kinase C and transition metals but are inhibited by calpain antagonists

Several cascades downstream of the [Ca²⁺]_i rise were checked in search of possible mechanisms for the observed NO release. Cell incubation with the calmodulin antagonists W-7 (20 μM) and calmidazolium (1 μM) or with the protein kinase C (PKC) antagonist staurosporin (1 μM) failed to inhibit the ACh (10 μM)-induced changes in DAF-FM fluorescence (Figure 6A).

NO responses were also insensitive to a specific membrane-permeable heavy metal chelator TPEN (*N,N,N*-tetrakis(2-pyridylmethyl)ethylenediamine; 10 μM). By contrast, cell treatment with the calpain inhibitor PD150606 (100 μM), but not its ineffective analogue PD145305, substantially inhibited NO responses (Figure 6B). The same inhibition was also seen in the presence of substances chemically unrelated to PD150606 calpain inhibitors (applied as a mixture)—calpain inhibitor I (20 μM), loxistatin (30 μM) and calpain inhibitor III (100 μM).

Addition of purified μ- or m-calpain to high molecular weight fraction of crude pancreatic acinar cell lysate, which was preincubated with S-nitrosoglutathione (GSNO) or nitrosonium tetrafluoroborate, increased the rate of spontaneous S-nitrosothiol decomposition (Figure 6C). These data suggest that calpains can be involved in the intracellular redistribution of NO.

NOS-independent NO release in other cell types

Patch pipette loading with DAF-FM was applied to acutely isolated mouse dorsal root ganglia (DRG) neurons, bovine adrenal chromaffin cells and cells from bovine aortic endothelial line (BAE-1). In the continuous presence of NO inhibitors in the extracellular and intracellular solutions,

Figure 6 The role of calcium-dependent signalling cascades on ACh-induced NO release. **(A)** Effect of calmodulin inhibitors W7 and calmidazolium (yellow trace, $n = 12$), PKC inhibitor staurosporin (green trace, $n = 15$) and heavy metal scavenger TPEN (grey trace, $n = 12$). The black trace (same as in Figure 2E) shows control NO responses. **(B)** Effect of calpain inhibitor PD150606 (brown trace, $n = 9$) and its ineffective analogue PD145305 (pink trace, $n = 8$). Effect of non-thiol calpain inhibitors (type I, type III and loxistatin) (blue trace, $n = 16$) is shown. Control is shown by the black trace. The asterisks by the time axis indicate statistically significant ($P < 0.05$) difference at given time points between data from control and calpain inhibition by non-thiol-based antagonist experiments (blue asterisks, comparison between blue and black traces) and between PD150606 and PD145305 data (brown asterisks, comparison between brown and pink traces). Note that there is no statistically significant difference between the control (black trace) and ineffective analogue PD145305. **(C)** Calpain-induced release of NO from the pancreatic lysate. The time course of NO release from the lysate pretreated with GSNO. Arrows show the time when μ -calpain (10.1 U/ml) and HgCl_2 were added into the cuvette.

the adrenal chromaffin cells, challenged with 100 μM ACh, developed fluorescence responses only in four out of 22 experiments (Supplementary Figure S2A). Stimulation of Ca²⁺ entry with 50 mM KCl resulted in a rise of cell fluorescence in a small proportion (three out of 12) of adrenal chromaffin cells (Supplementary Figure S2B); however, a majority (five out of seven) of DRG neurons responded to 50 mM KCl (Supplementary Figure S2C). In patched BAE-1 cells pretreated with 7-NI and L-NAME, stimulation with 100 μM ATP evoked resolvable fluorescence rise in three out of four cells (Supplementary Figure S2D). These experiments indicate that the calcium-dependent NO release from S-nitrosothiols is not restricted to pancreatic acinar cells and may be a more general phenomenon.

Discussion

The main finding in this study was the demonstration that a high $[\text{Ca}^{2+}]_c$ leads to liberation of NO in pancreatic acinar cells. We were able to resolve NO responses to supramaximal ACh stimulation in 28% of the intact cells (with unperturbed cytosol) as well as in 67% of the dialysed cells. We ascribe the difference to endogenous buffering of RNS by cytosolic GSH. This idea was supported by experiments with GSH depletion (Figure 1C) and addition of a high amount of exogenous GSH into the intracellular medium (Figure 3A). We found that pancreatic acinar cells rapidly release NO owing to its displacement from the S-nitrosothiol pool, where NO is reversibly bound to certain cysteine residues, rather than its *de novo* synthesis by NOS. This conclusion is based on two lines of evidence. First, NOS blockade made a statistically significant difference to the NO responses, but only following a considerable delay after start of the ACh application. During the first 200 s, the averaged traces for control cells and cells with inhibited NOS were virtually identical (Figure 3C), whereas at the end of the recordings there was a statistically significant difference. Thus, it seems unlikely that NOS is involved in the initial part of the NO responses but it could contribute up to 50% of the response by the end of the recording. Second, cell treatment aimed at increasing the amount of S-nitrosothiols upregulated the NO responses and the effect became statistically significant relatively early (Figure 3C). We also found that cells possessed a substantial amount of high molecular weight S-nitrosothiols, resistant to wash out by the patch pipette. This is in line with the notion that most of the intracellular S-nitrosothiols are high molecular weight proteins, whereas small S-nitrosothiols ($M_r < 5000$) are usually kept at a very low level (Liu *et al*, 2001). From experiments shown in Figures 3, 4A and B, we estimate that approximately 3 μM of S-nitrosothiols is denitrosylated in a calcium-dependent manner during supramaximal ACh stimulation. This implies quite high concentrations of reversibly S-nitrosylated proteins in these cells, which are expected to change their properties owing to denitrosylation (Hess *et al*, 2005). In our experiments, the NOS-independent NO changes were also found in DRG neurons, suggesting that this could be a general mechanism of NO signalling. It is interesting to note that a substantial NOS-independent release of NO can be triggered repeatedly in several types of smooth muscles by UV light (Furchgott *et al*, 1985). Such a photorelaxation phenomenon indicates that internal NO stores in smooth muscle cells must have a considerable capacity (Venturini *et al*, 1993); however, the ability for such stores to release NO under physiological conditions has not been shown previously. In our study, we demonstrate that a physiological stimulus—cytosolic calcium rise—can release NO from a store in a calpain-dependent manner.

The ability of pancreatic acinar cells to take up and release NO may be important in maintaining a background level of NO in the pancreas. A low constitutive presence of NO was shown to protect acinar cells against adverse stimuli (Molero *et al*, 1995; Werner *et al*, 1998; Jaworek *et al*, 2000; Andrzejewska *et al*, 2002; Sanchez-Bernal *et al*, 2004). In line with this, we observed in our experiments a relatively low and delayed ACh-stimulated NOS activity in the acinar cells. It is possible that, like in other exocrine cell types,

a [Ca²⁺]_c increase alone was not enough to fully activate NOS, and an additional specific (non-muscarinic) receptor stimulation is needed (Looms *et al*, 2002). Thus, some optimal, spatial and temporal, profile of the NO concentration in the pancreas should exist, and the acinar cells could be essential in maintaining this by serving as both NO releaser and NO buffer. For example, the acinar cells may protect the islet cells, which express more NOS but are highly vulnerable to nitrosative stress (Kroncke *et al*, 1993), by scavenging the RNS in the vicinity of the islets. The amplitude and time course of NO responses showed substantial heterogeneity across the cells and this may reflect differences in the S-nitrosothiol pool capacity, for example, because of different cell locations in the pancreas in relation to prominent NO sources (nerve endings, endothelial cells, etc.) before the cell isolation. The reason for the variability in intact (non-patched) cells can also be differences in the capabilities of these cells to bind NO or other free radicals (e.g. owing to different levels of GSH). The strong increase in the DAF-FM responses following GSH depletion confirms the important role of this thiol as a buffer for RNS in pancreatic acinar cells. This also suggests that in intact cells, during the secretagogue-induced Ca²⁺ rise, the major part of the NO released from the high molecular weight S-nitrosothiols binds to GSH (and consequently only a small amount of NO can escape the cell). From the results of the amperometric experiments (see Supplementary Figure S1B), we estimate that supramaximal ACh stimulation should induce an approximately 100 nM increase in tissue concentration of NO (in free radical form). For this estimation, we assumed that the cell density in our amperometric experiments is $5 \times 10^6 \text{ ml}^{-1}$, the average cell volume is $3 \times 10^{-9} \text{ ml}$ and that ~80% of the pancreas is occupied by acinar cells.

There is growing evidence that nitrosylation/de-nitrosylation of proteins can dynamically occur *in vivo* and can affect their function. NO group exchange between S-nitrosothiol and thiol molecules, which can occur by means of oxygen-independent reversible S-transnitrosation reactions, was shown to be regulated by pH (Liu *et al*, 1998) and trace copper (Tao *et al*, 2002). Protein S-nitrosothiol decomposition giving rise to NO or low molecular weight intermediate S-nitrosothiols can also be enzymatically catalysed by thio-reductase (Nikitovic and Holmgren, 1996) and disulphide isomerase (Sliskovic *et al*, 2005) as well as by some uncharacterized copper-containing enzymes (Gordge *et al*, 1996). It was also reported that calcium could directly regulate binding of NO to tissue transglutaminase (Lai *et al*, 2001) and calbindin D28 (Tao *et al*, 2002). The results of our experiments suggest that calcium can regulate denitrosylation of proteins in pancreatic acinar cells. NO in turn is known to regulate calcium signalling (Clementi, 1998), including stimulation of calcium entry (Xu *et al*, 1997; van Rossum *et al*, 2000), activation of ryanodine receptors (Eu *et al*, 2000) and so on. This suggests additional possibilities for feedback regulation of NO and calcium signalling cascades.

Our experiments indicate that a cytosolic calcium rise itself (without the production of other second messengers by secretagogues) is sufficient to induce a NO increase. To our surprise, inhibition of the ubiquitous calcium-regulated proteins calmodulin and PKC had no effect on NO responses. The membrane-permeable metal chelator TPEN was also without effect, indicating that the possible catalysis of NO release by

transition metals (which might be liberated into the cytosol during Ca²⁺ signals) was not occurring. The results from the experiments with calpain inhibitors suggest that this protease could be a mediator of the effect. This was further confirmed by experiments in which calpain facilitated NO release from pancreatic lysate (Figure 6). It is important to note that the expression of μ - and m-calpain in the exocrine pancreas was previously established (Weber *et al*, 2004). The putative mechanism by which calpains release NO is that calpain-induced partial proteolysis of certain S-nitrosylated proteins may reduce the stability of the S–NO bond and promote thiol denitrosylation. The list of known proteins subjected to both S-nitrosylation and calpain degradation includes annexin II, creatine kinase, caspase-9, src kinase, epidermal growth factor receptor, ryanodine receptor-1, poly(ADP)ribose polymerase, some PKC isoforms and calpains themselves. Some of these proteins may be constitutively expressed at high levels.

This study is the first attempt to measure NO in single live pancreatic acinar cells using fluorescent NO probes. We found that intracellular dialysis in the whole-cell patch clamp mode greatly improved the sensitivity of the NO detection, most probably because it removed mobile cytosolic nitrosation scavengers. The best sensitivity of NO detection was observed when the cells were loaded with the dye DAF-FM via a patch pipette. Such a loading approach can be advantageous for future studies of NO metabolism, especially in cells showing poor retention of DAF indicators. In this respect, the artificial removal of mobile free radical scavengers (most importantly, GSH) by patch pipette dialysis might have helped us to visualize physiological NO responses otherwise invisible with loading of DAF indicators into intact cells (Figures 1 and 2).

This paper reports NO production in pancreatic acinar cells. Importantly, the initial component of the calcium-induced NO signals was found to result from NO release from endogenous S-nitrosothiols and not owing to *de novo* production by NOS. This is a novel mechanism for generating NO responses.

Materials and methods

Cell preparation, dye loading and chemicals

Pancreata were obtained from adult male mice (CD1) humanely killed in accordance with the Animals (Scientific Procedures) Act, 1986. The pancreatic acinar cells were isolated by digestion with purified collagenase (200 U/ml) as described previously (Park *et al*, 2001) and used within 4 h. Crude cell lysate was obtained by homogenization of the whole pancreata in the presence of protease inhibitor cocktail. Before the experiments, the lysate was passed through a PD-10 column (Amersham, Pollards Wood, UK).

DRG neurons were obtained from adult male mice (CD1) by digestion with purified collagenase (400 U/ml) and trypsin (0.5 mg/ml) and used within 6 h. Bovine adrenal chromaffin cells were isolated and cultured up to 4 days in professor R Burgoyne's laboratory (Constable *et al*, 2005). BAE-1 culture was obtained from European Collection of Cell Cultures (ECACC, UK).

Imaging experiments were performed at room temperature (20–22°C). All experiments were performed under continuous superfusion of extracellular solution containing (in mM) the following: NaCl, 140; KCl, 4.7; MgCl₂, 1.13; CaCl₂, 1; glucose, 10; HEPES-NaOH, 10 (pH 7.3) and L-arginine 100 μM . For the experiments with DRG neurons, the extracellular solution was modified to contain 2 mM CaCl₂. Agonists and antagonists were added to the extracellular solution at the stated concentrations. DAF-FM was from Molecular Probes (Eugene, OR, USA); DTCS, Angeli's

salt, MAHMA NONOate, diaminorhodamine-4M, glyco-SNAP (II), calpain I and II (μ - and m-calpains, respectively), calpain inhibitor set and PD145305 were from Calbiochem (Nottingham, UK); EDTA-free protease inhibitor tablets were from Roche (Welwyn Garden City, UK); collagenase was from Worthington Biomedical Corp. (Lakewood, NJ, USA) and all other chemicals were purchased from Sigma-Aldrich (Poole, UK). DAF-FM T was made by preincubation of DAF-FM with excess of Angeli's salt. Fe²⁺-DTCS stock was made by addition of two times excess DTCS to FeSO₄ and used immediately. Fresh stock solution of sp/NO in 10 mM NaOH was prepared daily and stored on ice in darkness. Loading/staining of cells with DAF-2 DA was achieved by incubation of cells/tissue with the 2 μ M of the dye in darkness at 37 °C for 30 min.

Imaging

Fluorescence images were obtained using Nikon Diaphot inverted fluorescence microscope (Nikon Ltd, Kingston, UK). Cells were illuminated by 480 nm light from a monochromator using a $\times$ 100 1.3 NA objective lens. The emission light was collected at wavelengths longer than 500 nm. Images were digitized, averaged and analysed using a Quanticell-700m imaging system from Visitech International (Sunderland, UK).

Electrophysiology and solutions

Single isolated cells or individual cells in small clusters were dialysed with intracellular solution, using the standard whole-cell patch clamp configuration with micropipettes of resistance 2–3.5 M Ω made from borosilicate glass capillaries (GC150TF-7.5, Harvard Apparatus Ltd, Kent, UK). EPC-7 amplifier (Heka Elektronik, Lambrecht/Pfalz, Germany) was used. The dye-free intracellular solution contained the following: KCl 140 mM; Na-HEPES 10 mM; Mg-ATP 4 mM; MgCl₂ 1.13 mM; EGTA 0.2 mM (pH 7.3). The DAF-FM-containing intracellular solution included the following: KCl 120 mM; Mg-ATP 4 mM; MgCl₂ 1.13 mM; EGTA 0.2 mM; Na-HEPES 10 mM; DMSO 0.4%; DAF-FM 47 μ M (pH 7.3). High calcium buffering DAF-FM-containing intracellular solution included the following: KCl 120 mM; Na- Mg-ATP 4 mM; MgCl₂ 1.13 mM; CaCl₂ 2 mM; BAPTA 10 mM; DMSO 0.4%; DAF-FM 47 μ M; HEPES 10 mM (pH 7.3). For the perforated patch experiments, the DAF-FM-containing pipette solution additionally contained β -escin 20 μ M, FAD 10 μ M, FMN 10 μ M, BH₄ 5 μ M, NADPH 100 μ M and L-arginine 100 μ M. Cell-free experiments were conducted in solution made of KCl 140 mM, Na-HEPES 10 mM, MgCl₂ 1.13 mM, EGTA 0.2 mM and DAF-FM 47 μ M (pH 7.3).

During electrophysiological experiments, the cells were held at –30 mV potential, unless indicated otherwise, with repetitive short test pulses to 0 mV, and Ca²⁺ signals were reported by the calcium-dependent chloride current (Petersen, 1992) at –30 mV. The data collection commenced 10 min after establishing the 'whole-cell' mode. For high potassium stimulation experiments, the cells were held in the zero-current mode.

S-nitrosothiol detection

The measurements of the total cell S-nitrosothiol content (Figure 4, inset) were made using fluorometric assay based on Saville reaction at physiological pH, essentially as described by Cook *et al* (1996). In order to reduce the effect of protein autofluorescence, 2,3-diaminonaphthalene was substituted in our experiments by a longer wavelength indicator diaminorhodamine-4M. Every cell preparation was split into six equal cell samples, and the concentration of S-nitrosothiols in test samples was expressed as a percentage of control. After test incubations, cells were washed twice with intracellular-like solution to eliminate the traces of NO donors. Then, cells were lysed by Triton X-100 (0.1%) in the presence of diaminorhodamine-4M (50 nM) and HgCl₂ (10 mM). The fluorescence was monitored using Perkin-Elmer LS50B fluorescence spectrometer at 545 nm excitation and 575 nm emission wavelengths.

The *in vitro* measurements of the kinetics of S-nitrosothiol decomposition (Figures 4B and 6C) were conducted using DAF-FM (485 nm excitation and 530 nm emission wavelengths).

Amperometric measurement of NO

The measurements were performed using ISO-NOPMC sensor (WPI Ltd, Aston, UK) connected to ISO-MO Mark II meter (WPI). The analog signal was digitized using a DUO-18 data acquisition system (WPI). The calibration was made using MAHMA NONOate.

Data analysis

The fluorescence of the cells during the control period was extrapolated and used to compensate for the flux of the indicator from the pipette, leakage and bleaching, and this extrapolation was then applied to the whole time span. The normalized responses were obtained by dividing the traces of fluorescence by such extrapolation curves. Results are reported as means $\pm$ s.e.m. for the indicated number (*n*) of experiments. In Figures 1C, 2C and 3A–C, error bars for clarity are shown for every third point. Statistical comparisons were performed with the Student's *t*-test. Statistical significance was assumed for *P* < 0.05.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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