A role for Nitric Oxide-mediated glandular hypofunction in a non-apoptotic model for Sjögren's syndrome.

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Abstract

Objective

To investigate a role for the inflammatory mediator, nitric oxide (NO) in Sjögren's syndrome, an autoimmune condition characterised by salivary and lacrimal gland hypofunction resulting from failure of acinar cells to secrete.

Methods

FURA-2 microfluorimetry was used to measure agonist evoked changes of $[Ca^{2+}]_i$ in isolated mouse and human salivary acinar cells following exposure to NO donors.

Results

NO had a biphasic effect on salivary acinar function. Acute exposure to NO (2 minutes) caused a cyclic GMP-dependent, ODQ-sensitive increase in the Ca^{2+} signal elicited in response to ACh stimulation, consistent with stimulation of Ryanodine receptors by cyclic ADP ribose. Prolonged exposure to NO (>40 minutes) significantly reduced the ACh-evoked Ca^{2+} signal by a mechanism independent of cyclic GMP. We found no differences between the responses of human and mouse acinar cells.

Conclusion

Our data show that chronic exposure to NO, which is known to be elevated in Sjögren's syndrome, could have a role in salivary gland hypofunction. We note a similarity in the response to stimulation of salivary acinar exposed to NO and that which we have previously reported in salivary acinar cells isolated from patients with Sjögren's syndrome. We speculate that NO mediated nitrosylation of one or more elements of the signal transduction pathway could underlie down-regulation of salivary function in Sjögren's syndrome.

Introduction

Sjögren's syndrome is an autoimmune condition in which salivary glands lose the capacity to secrete saliva. For many years, it has been assumed that secretion declined along with the physical atrophy of the glands, which is clearly apparent by the end stages of the disease. More recently, it has been demonstrated that the loss of function precedes the loss of glandular tissue and that many patients who are incapable of saliva production nevertheless have histologically normal acinar tissue that is functional *in vitro* [1]. Glandular hypofunction cannot be a consequence of atrophy in these patients as their glandular tissue has not atrophied.

We have proposed an alternative, non-apoptotic model to account for glandular hypofunction in Sjögren's syndrome in which lack of function follows interaction between the autoimmune response and stimulus-secretion coupling [2]. Exploration of this model requires some understanding of the mechanisms of stimulus secretion coupling, the "core" elements of which are widely understood to be: (a) activation of G_q-protein coupled muscarinic M3 acetylcholine receptors; (b) an increased production of inositol 1,4,5 trisphosphate (IP_3); (c) IP_3 mobilisation of Ca^{2+} from intracellular stores; and (d) activation of an apical membrane Cl^- channel in response to the increase in $[Ca^{2+}]_i$ Fluid secretion is driven by Cl^- efflux across the apical membrane of the acinar cells [3].

In principle, any aspect of the immune response that prevented production of IP_3 and or Ca^{2+} mobilisation is a candidate for a pathogenic role in Sjögren's syndrome. In fact, the range of potential antisecretory agents is much wider, because stimulus-secretion coupling is more complex than the simple description of the "core" elements would suggest [4, 5]. Effective stimulus-secretion coupling does not necessarily follow *any* Ca^{2+} signal, but rather requires an *appropriate* signal in an

appropriate place at an *appropriate* time [5]. For example, whereas a small brief increase in $[Ca^{2+}]_i$ at the apical pole of the cell might be sufficient to trigger secretion, the same signal at the basolateral pole would likely not [6]. The loss of secretory function seen in Sjögren's syndrome does not therefore necessitate a complete abolition of stimulation-evoked increases in either IP_3 or $[Ca^{2+}]_i$ but could rather be a consequence of any factor which perturbed the orderly origin and progression of the Ca^{2+} signals.

One element of the signal transduction cascade with a key role in ensuring that increased IP_3 generates an appropriate Ca^{2+} signal is calcium-induced calcium release (CICR). CICR is a positive feedback process that amplifies Ca^{2+} signals and which depends on the Ca^{2+} sensitivity of the intracellular Ca^{2+} release channels. In salivary acinar cells, Ca^{2+} release from intracellular stores is via both an IP_3 receptor and a ryanodine receptor [4], both of which have similar Ca^{2+} sensitive properties. Whereas the IP_3 receptor, activated by IP_3 in response to ACh receptor stimulation, is the trigger for the Ca^{2+} signal, Ca^{2+} release through the ryanodine receptor is thought to "shape" the signal and to have a role in determining the magnitude and time course of the response. The contribution of the ryanodine receptors to Ca^{2+} signalling is regulated by cyclic ADP ribose concentration which is itself be regulated by cGMP levels [7].

Any factor that regulates cGMP and or cADP ribose levels has therefore the capacity to alter stimulus-secretion coupling independently of any direct effect on IP_3 production or on IP_3-dependent Ca^{2+} release. One such factor is Nitric Oxide (NO), which activates guanylate cyclase and stimulates cGMP production.

In terms of our model for secretion, increased NO levels should amplify the ACh-evoked Ca^{2+} signal and enhance fluid and electrolyte secretion. However, exactly the

opposite has been observed in Sjögren's syndrome [8] where fluid and electrolyte secretion are inhibited despite elevation of NO levels. We have therefore mimicked this aspect of Sjögren's syndrome *in vitro* by exposing salivary acinar cells to NO so that we can determine whether the agonist-evoked Ca^{2+} signal is amplified by NO, the prediction of the model, or inhibited, the observation of glandular pathology.

It is always much easier to perform experiments using cells from a mouse model rather than human cells, mouse acinar cells may easily be obtained from an age and sex matched population. It is *possible* to obtain equivalent cells from humans, but not *easy*. As a consequence, most of the previous studies modelling Ca^{2+} signalling and fluid secretion have involved mouse salivary acinar cells, and it has been assumed that equivalent observations could be made in human cells, were they available. We think it important to show directly whether there are strong qualitative similarities in the responses of human and mouse cells particularly as we are examining a pathological processes.. . In this study, we have used mouse submandibular acinar cells to prototype experimental protocols and then applied selected successful protocols to human submandibular acinar cells. We present the data from both mouse and human cells so that we can see how well the mouse model mimics the human situation.

Methods

Solutions

The extracellular bathing solution contained in mM: 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 1 $CaCl_2$, 10 glucose buffered to pH 7.4 with 10mM HEPES

The acinar cell culture medium: serum free 50:50 Dulbecco's MEM:F12 medium plus antibiotics and antimycotics (Life Technologies UK)

Mouse Acinar cell collection

Adult male CD1 mice were killed by cervical dislocation and submandibular acinar cells were isolated by collagenase (100units/ml, Worthington Diagnostic USA) digestion in extracellular media containing 1mM Ca^{2+} as described previously [9]. Briefly: glands were removed from the animal, stripped of surrounding capsule, injected with collagenase and incubated for 15-20 minutes in a shaking water bath at 35^{o}C. Collagenase was removed by centrifugation and cells were further dispersed by mechanical agitation to produce a small number of individual cells and a larger number of small clumps (2-8 cells).

Human Acinar cell collection

Following local ethical approval (Sefton LREC, EC.38.02) and informed written consent, small portions of human submandibular glands were collected, at the time of surgery, from patients undergoing submandibular gland removal as part of routine head and neck surgery. The small size of the gland portion collected for research did not interfere with the subsequent diagnosis and treatment of the patient. All collected samples were transported to the laboratory on ice in 'acinar cell culture media' within 1 hour of removal. Acinar cells were isolated from the human tissue using the same techniques employed to isolate mouse acinar cells [9].

Acinar Cell preparation

Following dispersal, cells were suspended in 'Acinar cell culture media' and placed onto circular glass coverslips (22mm diameter) coated with a thin (≈1 mm) layer of a basement membrane matrix (Matrigel, Becton Dickinson, UK) 25. Each coverslip was placed into one well of a 6 well plate and covered with 'Acinar cell culture media' and kept overnight at 37^{o}C with 5% CO_2.

Microfluorimetry

Cells were removed from culture immediately before an experiment and loaded with Fura-2 by incubation for 20 minutes in the presence of 2 μM of cell permeable fura-2 acetoxymethylester (Fura-2 AM, Molecular Probes). The acinar cell coated coverslips formed the base of a perfusion chamber placed on to the stage of an inverted microscope (TMD 100, Nikon, Kingston, Surrey, UK). All experiments were carried out at 24 ± 2^{o}C. Measurements were made using 1000x magnification on single cells, either completely isolated or part of a small (2-8) cell clump. Cells were superfused continuously at 0.5 ml/min from one of several parallel superfusion pipettes.

The ratio of UV light emitted at 510nm following excitation at 340nm to that emitted following excitation at 380nm was measured using a Cairn (Cairn Research Ltd, Faversham, Kent, UK) spectrophotometer (Excitation was at 96Hz, data were averaged online and collected at 4Hz.). Intracellular Ca^{2+} activity was calculated from this ratio using the Grynkiewiez equation and custom written software. Averaged changes in Ca^{2+} were calculated from the plateau phase of the Ca^{2+} signal.

NO Donors and Inhibitors

Cells were exposed to NO by perfusion with two chemically different donors: S-Nitroso-N-penicillamine (SNAP, 100-200 μM) and 1-Hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC-12, 200 μM) each with long half-lives for NO

release (approximately 10 hours & 5 hours at 22^{o}C for SNAP and NOC-12 respectively. Guanylate cyclase activity was inhibited using 1-H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1μM). Ryanodine receptor activity was inhibited using ryanodine (10μM)

Statistics

Numerical data are presented as mean ± Standard Error of the mean (number of observations). Significance was determined using Student's t test.

Results

We have designed a protocol to show whether exposure to NO affects the ACh-evoked Ca^{2+} signal. The most easily quantifiable Ca^{2+} signal is a small steady-state increase in response to a low level of ACh stimulation. Figure 1A shows the response of mouse submandibular acinar cells to repeated stimulation with 50nM ACh. The resultant increase in Ca^{2+} is highly reproducible in any one cell. Figures 1B and C show that exposure to NO released by the donors SNAP (100 µM) and NOC-12 (200 µM) respectively, increased the response to ACh. On average, under control conditions, the response to the third application of ACh was 103.7 ± 2.9 % that of the first (n=13). When exposed to SNAP after the second application of ACh, the response to subsequent stimulation rose to 226.5 ± 30.9 % of that before application of SNAP (n=7). Similarly, exposure to NOC-12 increased the response to 168.2 ± 12.8 % (n=16) of that seen before application. The amplification induced by either NO donor was statistically significant at $P < 0.01$. In some experiments, particularly those where a higher concentrations of SNAP (>=200µM, data not shown) was employed, baseline Ca^{2+} also increased, as described previously by Looms *et al* [10]. In other experiments using lower concentrations of NO donor, the Ca^{2+} baseline remained constant.

Application of this protocol to human submandibular cells returned very similar results. Repeated application of 50nM ACh under control conditions caused some attenuation of the ACh-evoked Ca^{2+} signal in these cells (figure 2A), nevertheless a brief preincubation with NOC-12 (200 µM) resulted in amplification of the baseline response (figure 2B). On average, the third application of ACh gave an increase in Ca^{2+}, 97.4 ± 6.1 % (n=7) of that seen in response to the first application of ACh.

Following exposure to NOC-12, the increase was 127.7 ± 9.1 (n=3). Compared to control, the amplification induced by NOC-12 was statistically significant at $P<0.05$.

These data show that NO amplified the ACh-evoked Ca^{2+} signal. We hypothesise that this amplification is mediated through cGMP stimulation of cADPr production following NO-stimulation of guanylate cyclase. Guanylate cyclase activity may be inhibited using 1-H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). The data in figure 3 (mouse submandibular cells) show that, in the presence of ODQ (1 μM), despite exposure to NO released by either SNAP (100 μM, figure 3A) or NOC-12 (200 μM, figure 3B) there was little amplification of the response to stimulation by 50nM ACh. On average, incubation in the presence of ODQ reduced the amplification caused by SNAP to 116.9 ± 3.7 % (n=4) of that prior to exposure to SNAP. This value is still significantly ($P<0.05$) greater than that seen in the absence of SNAP and ODQ but also significantly ($P<0.05$) reduced from the amplification seen in the presence of SNAP alone. The smaller amplification induced by NOC-12 was completely abolished by ODQ, where the response to NOC-12 and ODQ together was 106.1 ± 6.4 % (n=6) of that seen prior to application of NOC-12. This value is significantly ($P<0.01$) lower than that obtained in the presence of NOC-12 alone. Acute application of ODQ alone (1μM, data not shown) had no detectable effect on the baseline Ca^{2+} signal.

The proximate cause of NO-cADPr-mediated amplification of the ACh-evoked Ca^{2+} signal is activation of ryanodine receptors. This activation may be inhibited using ryanodine. The data in figure 3C (mouse submandibular cells) show that the ACh-evoked Ca^{2+} signal in the following co-incubation with ryanodine (10 μM) and NOC-12 (200 μM) was little different from that seen prior to exposure to the NO donor. The average response in the presence of ryanodine and NOC-12 was 129.8 ± 11.1 % (n=21) of that seen prior to exposure to NOC-12. This value is not significantly

different from that seen under control conditions and it is significantly lower ($P<0.05$) from that seen in the presence of NOC-12 alone. Acute application of ryanodine (10μM) had no detectable effect on the baseline Ca^{2+} signal (data not shown)

The averaged data from the sequence of experiments represented by example in figures 1 and 3 are summarised in figure 4 which shows that in mouse submandibular cells, the NO-dependent amplification of the ACh-evoked Ca^{2+} signal was inhibited by both ODQ and by ryanodine.

The data in figure 5A show that repetitive application of ACh to mouse submandibular acinar cells produced a consistent response over an extended period of time (>40 minutes). Thus the protocol may be applied to investigate the effects of more chronic exposure of the cells to NO. As may be seen in figure 5B, in the continued presence of SNAP (100 μM) for 40 minutes, the initial amplification of the ACh-evoked Ca^{2+} signal (*cf* figure 1) was followed by a progressive loss of responsiveness to stimulation. A similar pattern was observed following chronic exposure to NOC-12 (200 μM), although a longer exposure was required to significantly reduce the ACh response. On average, the response to ACh alone following stimulation over a period of 30 minutes was 101.9 ± 15.8% (n=13) of that of the response to the first exposure to ACh. Over the same period, the response to ACh in the presence of SNAP dropped to 25.3 ± 9.5 % (n=7) of that prior to exposure to SNAP. Similarly, the response to ACh after a 40 minute exposure to NOC-12 was 46.9 ± 10.1 % (n=16) of that before exposure to NOC-12 (figure 5C). The reduction in response to ACh stimulation was statistically significantly different ($P<0.01$) in the presence of either NO donor compared to control. The data in figure 5C also show significant Ca^{2+} mobilisation by thapsigargin stimulation, following a 65 minute incubation in NOC-12, even though the response to ACh had dropped almost to zero. This last observation was typical of 6/7 similar experiments.

The response to 50nM ACh stimulation following a prolonged pre-incubation (2-5 hours) in the presence of 200 µM NOC-12 was measured (trace not shown) and found to be on average 49.7 ± 24.3% (n=9) of the averaged first response to ACh seen without preincubation. These data cannot easily be directly compared to those obtained using the repetitive stimulation protocol, however it is clear that the response to ACh is both lower than that seen without exposure to NOC-12 and also that prolonged exposure to NOC-12 alone did not completely abolish the response to ACh.

The data in figure 5D (mouse submandibular cells) show that application of the guanylate cyclase inhibitor ODQ (1 µM) had no effect on the inhibition of the ACh-evoked Ca^{2+} signal caused by prolonged exposure to the NO donor SNAP. The average response to ACh following 30 a minute incubation in the presence of ODQ and SNAP was 5.1 ± 3.4% (n=4) of that prior to incubation. This value is lower than that seen in the presence of SNAP alone. Similarly, incubation with ODQ did not prevent the reduction in the ACh response caused by NOC-12 (200 µM). On average, using the repetitive stimulation protocol, the response to ACh following a 30 minute co-incubation of NOC-12 and ODQ was 41.9 ± 15.2% (n=6) of that prior to incubation. We were unable to determine whether the chronic exposure to NO was affected by ryanodine because ryanodine itself caused significant inhibition of the ACh response (data not shown). We found no additive effects of ryanodine and NO donor together (data not shown).

Similar experiments were performed using human submandibular acinar cells. The data from human cells are slightly harder to interpret than those from the mouse cells because of the attenuation in the response to repetitive ACh stimulation seen in the

absence of NO donor (Figure 6A). This is most likely the result of slight overstimulation of these cells. Nevertheless, as may be seen in figure 6B, there was a more profound reduction in responsiveness induced by prolonged exposure to NOC-12 (200 μM). The average attenuation of the ACh response under control conditions was to 38.3 ± 7.7 % (n=6) of the initial response. In the presence of NOC-12, this fell to 15.2 ± 7.1 % (n=3) which represents a significant ($P<0.05$) reduction in response.

Discussion

NO donors have been previously shown to stimulate Ca^{2+} mobilisation in salivary acinar cells [10], most likely by enhancing ryanodine receptor (RyR) activity. We also found higher concentrations of NO donor to cause spontaneous Ca^{2+} release, in the absence of agonist stimulation (data not shown). Initiation of a Ca^{2+} signal is not thought to be the physiological role of Ca^{2+} release via RyR in acinar cells, nevertheless these data are consistent with studies in which infusion of a high concentration of cADPr, the endogenous RyR agonist, caused Ca^{2+} mobilisation [4]. Cyclic-ADPr stimulated Ca^{2+} mobilisation via RyR is not directly coupled to extracellular receptor activation and it is thought more likely that the role of cADPr and RyR is to modulate the ACh-evoked Ca^{2+} signal [4]. Our experimental protocol was designed to determine whether NO could be a regulator of this physiological role of RyR.

Our data show that, in both mouse and, for the first time, in human submandibular cells, exposure to NO donor enhanced the response to agonist stimulation (figures 1 & 2). This is most likely the result of NO release, rather than an artefact of a particular NO donor because similar enhancement was seen using two chemically unrelated NO donors, SNAP and NOC-12, and also with sodium nitroprusside (data not shown).

Inhibition by ODQ (figure 3) of the effect of the NO donor indicates that the enhancement was mediated through cGMP. Inhibition by ryanodine indicates involvement of RyR (figure 3). Together these data confirm the prediction of the model, that NO is able to up-regulate stimulus secretion coupling by increasing the contribution of Ca^{2+} release via RyR to the Ca^{2+} signal.

These data would seem to make a role for NO in the aetiology of Sjögren's syndrome less likely. However, the data in figure 5, where the effect of longer exposure to NO is examined, indicate that this is not the case. The enhancement of the ACh-evoked Ca^{2+} signal was short lived and vanished within 20-30 minutes. Furthermore, in both mouse and human submandibular cells, chronic exposure to NO ultimately rendered the cells less sensitive to ACh stimulation. The data in figures 5 and 6 show also that this is not a use-dependent artefact of ACh-stimulation. Mouse submandibular acinar cells showed almost no use-dependence in their response to ACh over the period of these experiments and, although there was some use- or time- dependence in the response of human acinar cells to repeated ACh stimulation, the decline in the response was significantly greater in the presence of NO donor. These data cannot be accounted for by the "Model for Secretion" (see introduction) but they are nevertheless consistent with the observation that NO levels are elevated in Sjögren's syndrome patients [8]. We have previously shown in labial glands from patients with Sjögren's syndrome that the concentration dependence of the ACh response is shifted to the right, i.e. these cells are less sensitive to stimulation [1]. This is exactly what we have observed in cells chronically exposed to NO.

The effect of chronic exposure to NO was not prevented by the guanylate cyclase inhibitor ODQ which suggests that the inhibitory effect is not mediated through cGMP. We have little evidence at present to indicate the mechanism by which NO inhibits stimulus-secretion coupling. The Ca^{2+} stores do appear to be still functional, inasmuch as Ca^{2+} may still be mobilised by the SERCA pump inhibitor thapsigargin after the response to ACh has diminished (figure 5C). Although, by itself a crude indicator, the thapsigargin data would suggest the effect of NO is not simply depletion of stored Ca^{2+}.

Whilst activation of soluble guanylate cyclase by NO binding to the haeme moiety of the enzyme remains the best understood mechanism by which NO affects cellular function [11], it is far from the only one. NO also contributes to both cellular physiology and pathophysiology through its role in triggering apoptosis, which is mediated through reactive nitrogen species (RNS) [12]. Furthermore, apoptosis is not an inevitable or even the only consequence of the production of RNS [13] and there is a growing body of evidence to indicate that reversible changes in the redox state of cysteine thiols can modulate many protein functions [14].

One protein in which the effects of nitrosylation have been extensively studied is the ryanodine receptor [15, 16] and it has been hypothesised that NO-mediated nitrosylation of RyR dubbed "redox signalling" [14, 17] could comprise an important regulator of the signal transduction process [18]. It is very difficult to predict with any certainty the impact that nitrosylation of the ryanodine receptor might have on stimulus-secretion coupling because the specificity with which cysteine residues are nitrosylated depends on many factors including the subcellular distribution of the target protein and the local chemical environment in which it is found [16, 18, 19]. Nevertheless, inhibition of RyR activity through nitrosylation could possibly account for our observations of an inhibition of the Ca^{2+} signal following prolonged exposure to NO donor.

We have no direct evidence that NO is down regulating RyR function through nitrosylation and furthermore, RyR is not the only component of the stimulus-secretion cascade that could be targeted by nitrosylation. Studies on RyR and many other proteins have identified concensus motifs likely to be nitrosylated [23, 24] and IP_3 receptors [25] also share these motifs. Store gated Ca^{2+} influx is also thought to

modified by NO [26, 27] and nitrosylation could also alter muscarinic receptor function by altering the distribution of receptors at the cell surface [28, 29].

Our observations are consistent with a role for NO in glandular hypofunction by a cGMP independent mechanism, possibly via S-nitrosylation of a key component of the stimulus-secretion cascade. These data could go some way towards explaining the right-shift in the ACh concentration dependence that we have seen in human labial gland cells from Sjögren's syndrome patients [1] because NO is known to be elevated in Sjögren's syndrome [8, 30-32]. Together these data support the concepts outlined in "A non-apoptotic model for Sjögren's syndrome" [2] and introduce mechanisms whereby not only amplification but also pathological inhibition of salivary secretion might be mediated by NO.

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Disclosure Statement

The authors have no financial interests, direct or indirect, that might affect, or be perceived to affect, the conduct or reporting of the work.

Key Points

1) Acute application of NO stimulates salivary secreting, most probably via increased cGMP and cADPribose leading to increased ryanodine receptor activity.
2) Chronic application of NO inhibits salivary secretion by an unknown mechanism, possibly nitrosylation of some element of the stimulus-secretion cascade.

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Figure Legends

Figure 1 The effect of acute application of NO donor on the ACh-stimulated Ca^{2+} signal in mouse submandibular acinar cells.

Intracellular $[Ca^{2+}]_i$ measured using fura-2 microfluorimetry in mouse submandibular acinar cells stimulated by successive application of ACh (50nM) for periods of approximately 60s.

A) In the absence of NO donor. B) Prior to application and in the presence of SNAP (200 µM). C) Prior to application and in the presence of NOC-12 (200 µM). The Ca^{2+} signal stimulated by ACh was amplified by exposure to either NO donor.

Figure 2 The effect of acute application of NO donor on the ACh-stimulated Ca^{2+} signal in human submandibular acinar cells.

Intracellular $[Ca^{2+}]_i$ measured using fura-2 microfluorimetry in human submandibular acinar cells stimulated by successive application of ACh (50nM) for periods of approximately 60s.

A) In the absence of NO donor. B) Prior to application and in the presence of NOC-12 (200 µM). The Ca^{2+} signal stimulated by ACh was amplified by exposure to NO donor.

Figure 3 The effect of acute application of NO donor, guanylate cyclase inhibitor and ryanodine receptor inhibitor on the ACh-stimulated Ca^{2+} signal in mouse submandibular acinar cells.

Intracellular $[Ca^{2+}]_i$ measured using fura-2 microfluorimetry in mouse submandibular acinar cells stimulated by successive application of ACh (50nM) for periods of approximately 60s.

A) In the continued presence of ODQ (1 µM) and prior to and following application of SNAP (200 µM). B) In the continued presence of ODQ (1 µM) and prior to and

following application of NOC-12 (200 µM). C) In the continued presence of ryanodine (10 µM) and prior to and following application of NOC-12 (200 µM).

The NO donor stimulated amplification of the ACh-stimulated Ca^{2+} signal was abolished by both ODQ and ryanodine. Neither ODQ or ryanodine themselves caused any inhibition of the ACh-stimulated Ca^{2+} signal.

Figure 4 Summary and averaged data showing the effects of SNAP, NOC-12, ODQ and ryanodine on the ACh-stimulated Ca^{2+} signal expressed as a percentage of the initial response to ACh.

These data are the average of 13, 4, 7, 6, 16 & 21 experiments , see text for values.

Figure 5 The effect of chronic application of NO donor on the ACh-stimulated Ca^{2+} signal in mouse submandibular acinar cells.

Intracellular $[Ca^{2+}]_i$ measured using fura-2 microfluorimetry in mouse submandibular acinar cells stimulated by successive application of ACh (50nM) for periods of approximately 60s.

A) In the absence of NO donor showing no use- or time- dependent change in the response over 40-60 minutes. B) Prior to application and in the presence of SNAP (200 µM). C) Prior to application and in the presence of NOC-12 (200 µM). The Ca^{2+} signal stimulated by ACh was inhibited or abolished by prolonged exposure to either NO donor. A Ca^{2+} signal was elicited by the SERCA inhibitor thapsigargin (2 µM) following loss of the response to ACh. D) In the continued presence of ODQ (1 µM) and prior to and following application of SNAP (200 µM). Inhibition of the ACh-evoked Ca^{2+} signal by prolonged exposure to NO donor was not prevented by the guanylate cyclase inhibitor.

Figure 6 The effect of chronic application of NO donor on the ACh-stimulated Ca^{2+} signal in human submandibular acinar cells.

Intracellular $[Ca^{2+}]_i$ measured using fura-2 microfluorimetry in human submandibular acinar cells stimulated by successive application of ACh (50nM) for periods of approximately 60s.

A) In the absence of NO donor showing some use- or time- dependent change in the response over 40-60 minutes. B) Prior to application and in the presence of NOC-12 (200 µM). The Ca^{2+} signal stimulated by ACh was inhibited or abolished by prolonged exposure to NO donor.

Figure 1

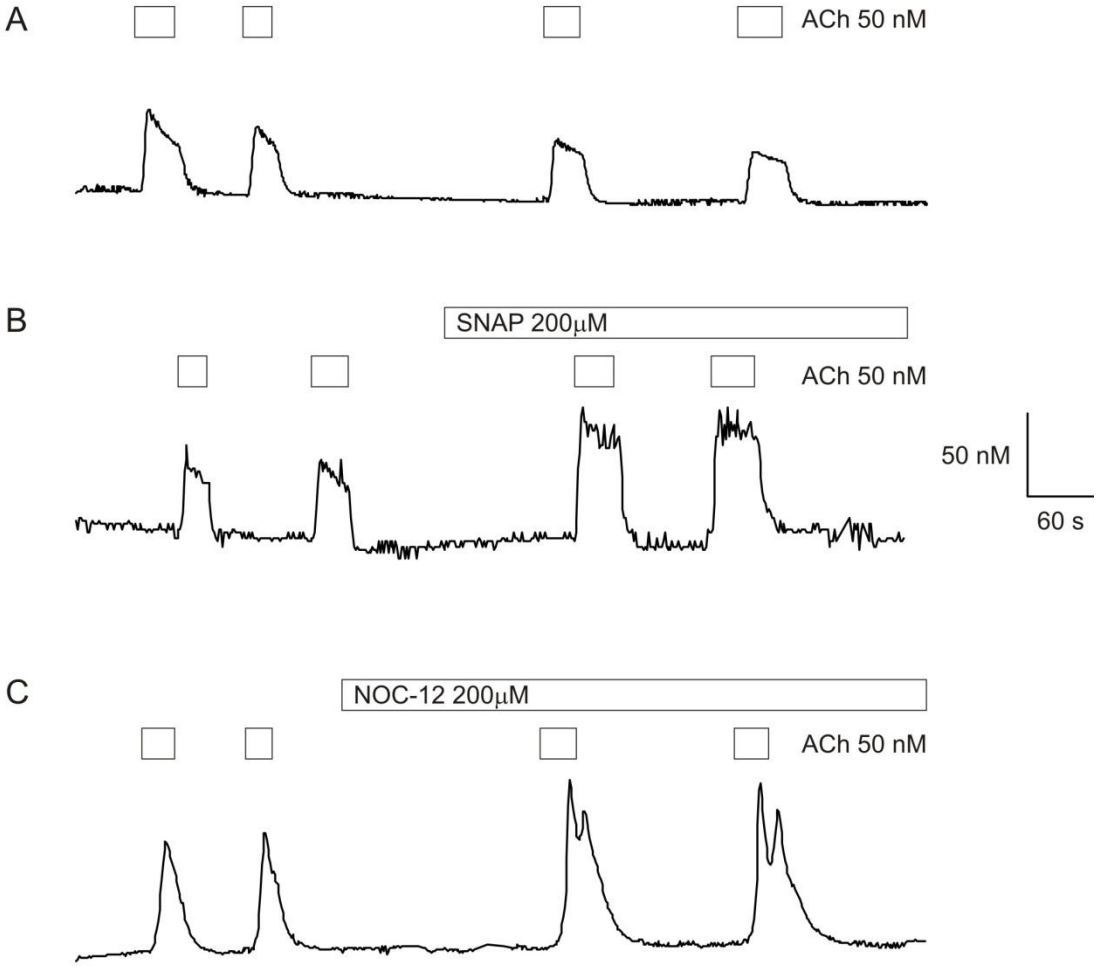
A
ACh 50 nM
B
SNAP 200μM
ACh 50 nM
50 nM
60 s
C
NOC-12 200μM
ACh 50 nM

Figure 2

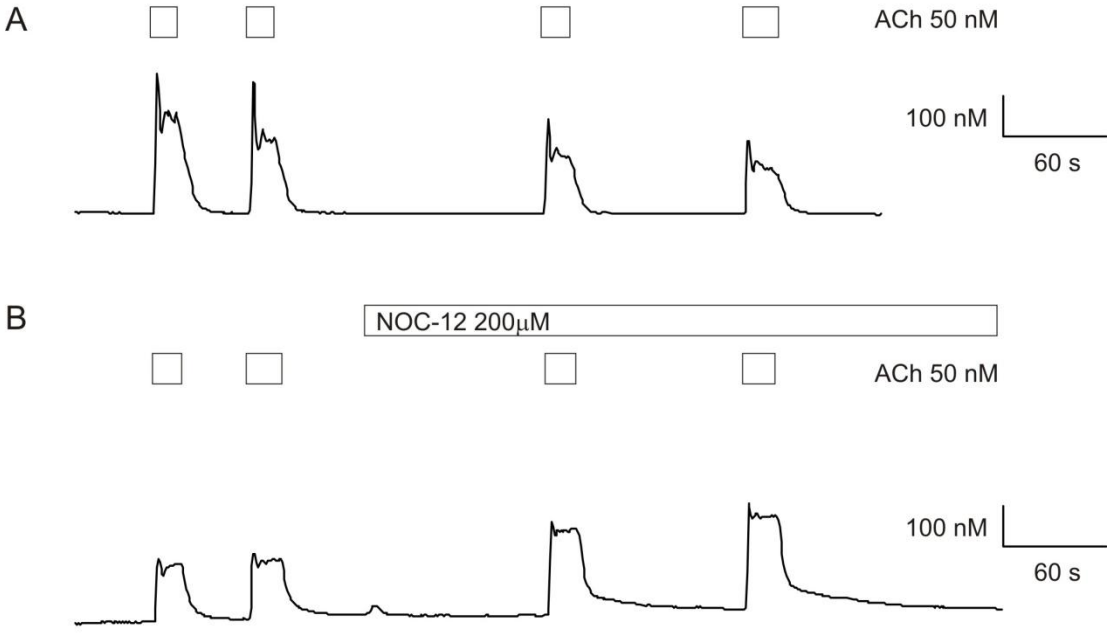

Figure 3

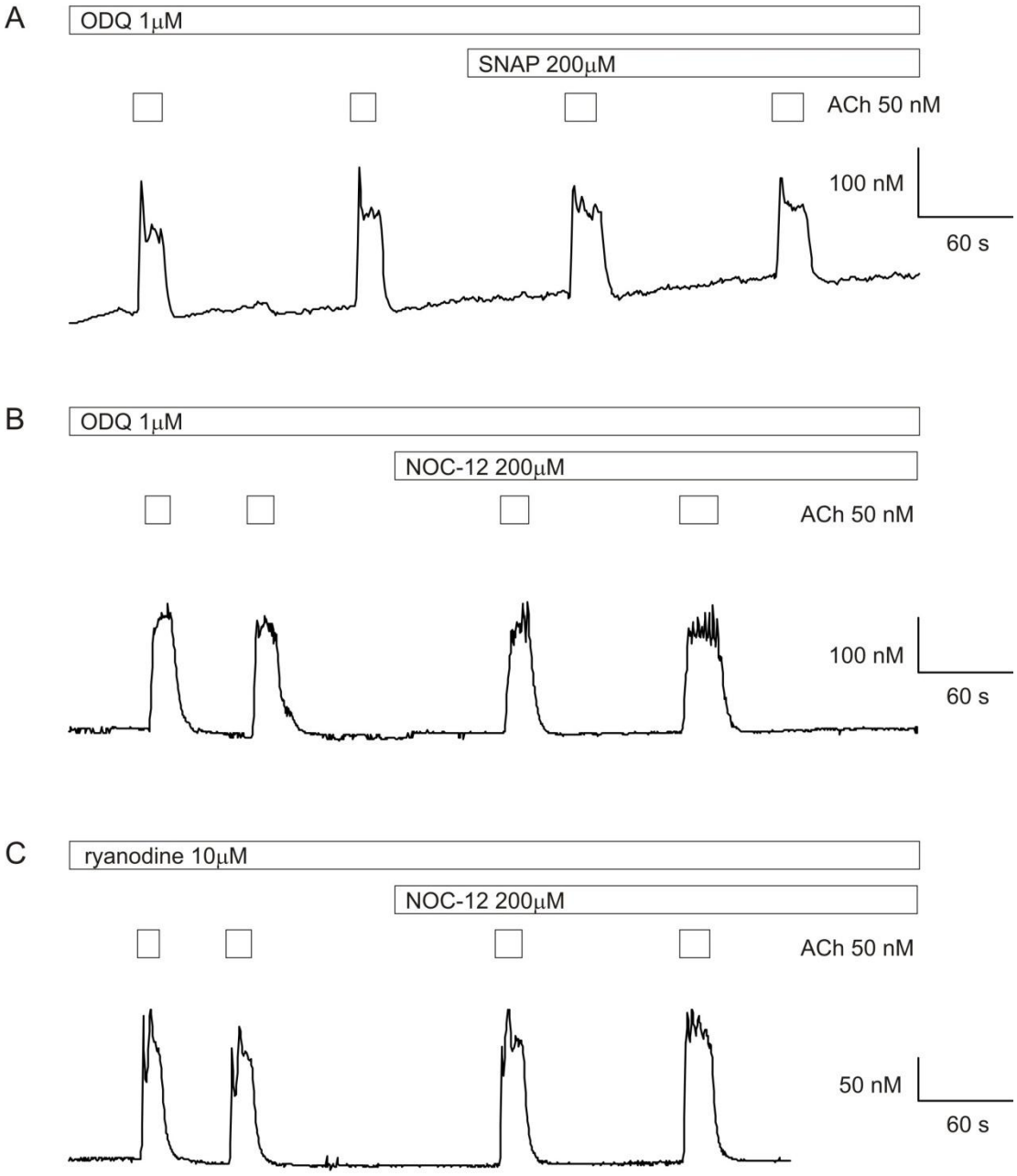
A
ODQ 1μM
SNAP 200μM
ACh 50 nM
100 nM
60 s
B
ODQ 1μM
NOC-12 200μM
ACh 50 nM
100 nM
60 s
C
ryanodine 10μM
NOC-12 200μM
ACh 50 nM
50 nM
60 s

Figure 4

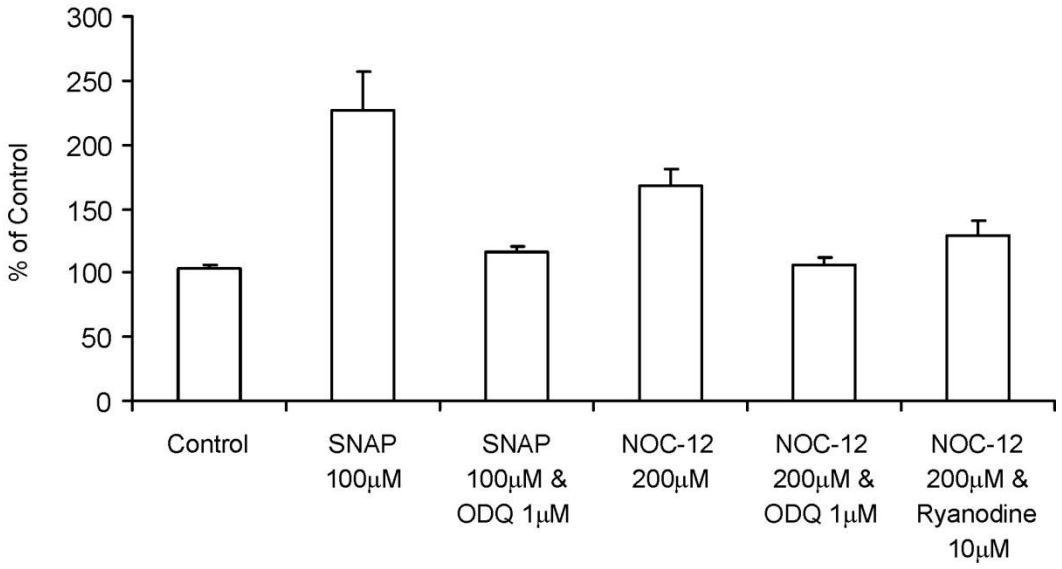
300
250
200
150
100
50
0
% of Control
Control
SNAP 100μM
SNAP 100μM & ODQ 1μM
NOC-12 200μM
NOC-12 200μM & ODQ 1μM
NOC-12 200μM & Ryanodine 10μM

Figure 5

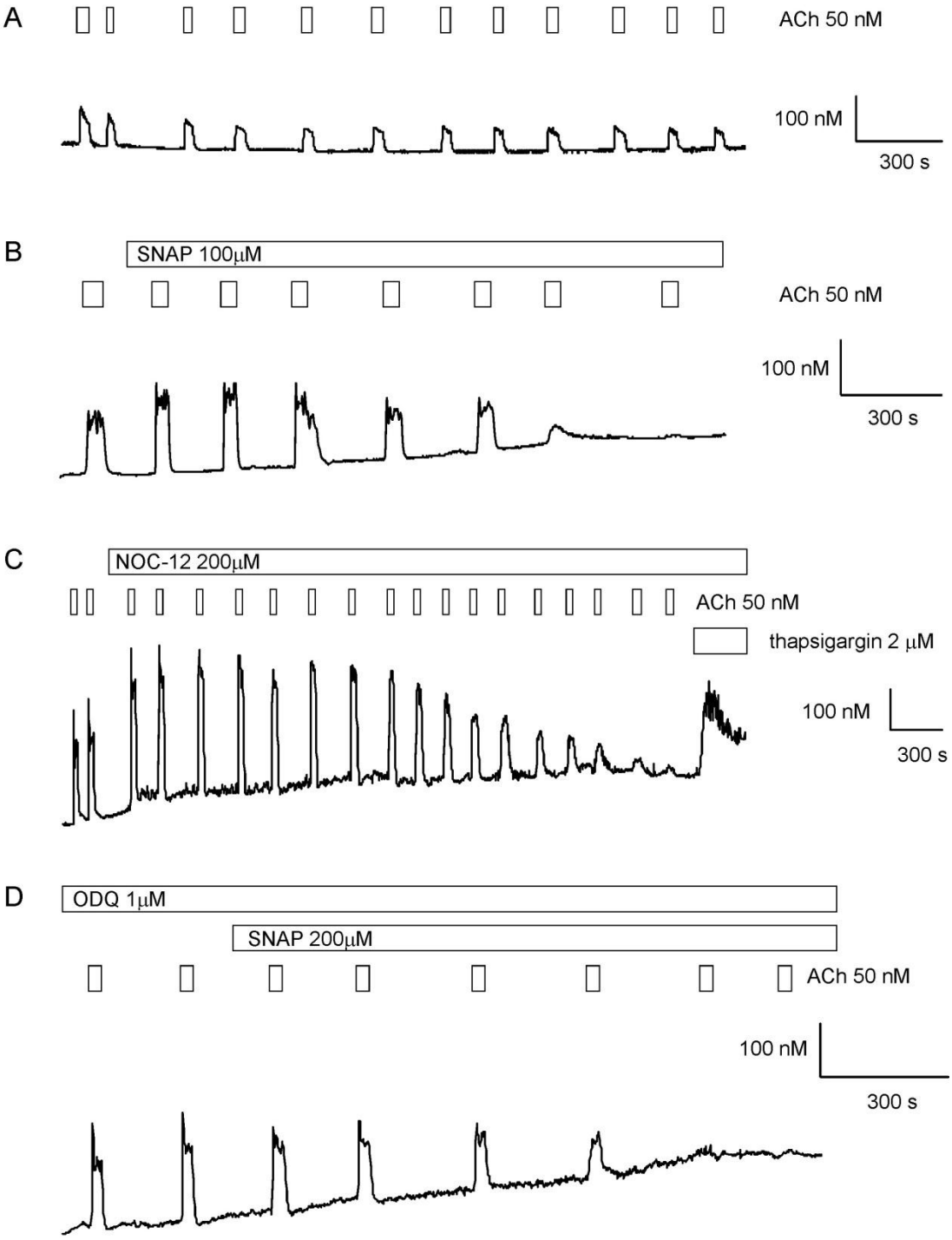
A
ACh 50 nM
100 nM
300 s
B
SNAP 100μM
ACh 50 nM
100 nM
300 s
C
NOC-12 200μM
ACh 50 nM
thapsigargin 2 μM
100 nM
300 s
D
ODQ 1μM
SNAP 200μM
ACh 50 nM
100 nM
300 s

Figure 6