Nitric oxide induces intracellular Ca^{2+} mobilization and increases secretion of incorporated 5-hydroxytryptamine in rat pancreatic β-cells

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Abstract **This study is the first to demonstrate that low concentrations of aqueous NO induce intracellular Ca^{2+} mobilization and an increase in secretory activity of rat pancreatic β-cells. Application of NO solution (2 μM) resulted in a transient increase in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of isolated cells, as assessed by video ratio imaging and single wavelength microfluorimetry. Amperometry revealed a simultaneous increase in the release of preloaded 5-hydroxytryptamine from the isolated cells. The NO-induced Ca^{2+} response primarily involves mobilization of endoplasmic reticulum Ca^{2+} stores, since the response was retained when cells were transferred to low Ca^{2+} medium, and completely inhibited when cells were pretreated with 10 μM thapsigargin. The Ca^{2+} response was also inhibited when cells were incubated with a high concentration of ryanodine (200 μM), suggesting that Ca^{2+} mobilization is via a ryanodine-sensitive store.**

Key words: Ca^{2+}; Nitric oxide; β-Cell

1. Introduction

Insulin secretion in pancreatic β-cells is stimulated by a variety of secretagogues, including D-glucose. Cell response is mediated by various signalling pathways, and is characterized by a rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which is functionally coupled to insulin secretion [1]. Nitric oxide (NO) is a potent mediator in intracellular signalling in a variety of cells [2]. Insulin secreting β-cells possess a constitutive NO-synthase [3], and are able to produce NO from L-arginine in response to D-glucose [3]. Hence, a physiological role for NO might be expected in β-cells. Since NO can generate cGMP through activation of a soluble guanylate cyclase [2], it is possible that NO may exert its effect partly, or wholly through an increase in cGMP within the cell.

Previous studies suggest that L-arginine and NO-donors generate NO and cGMP in β-cells [3,4] and that these species are modulators of insulin secretion. However, their effect on β-cell $[Ca^{2+}]_i$ has not been assessed, and their precise effect on insulin secretion remains controversial [3,4,5,6]. Studies suggest both stimulatory [3,4] and inhibitory [5] effects for NO on insulin secretion, and a stimulatory effect for cGMP [4]. Most studies to date have employed L-arginine and NO-donors such as sodium nitroprusside (SNP), in their assessment of secretory modulation by NO. Hence, NO production in many studies was only assumed, and possible non-specific effects attributable to NO-precursors were ignored. It is arguably more suitable to use low concentrations of gaseous NO, rather than higher concentrations of NO-precursors, in experiments designed to assess the signalling potential of NO. Owing to its short half life [7], a single application of native NO is expected to be rapidly oxidised and cleared from the bathing medium. Prolonged incubation of cells with high concentrations of NO-precursors may result in continuous delivery, and over-exposure of cells to NO, which is likely to be cytotoxic [7,8]. Furthermore, it is acceptable to presume that in a physiological system, a cellular response to NO might be rapid, and short-lived owing to cellular regulation of NO synthase (NOS) and NOs ephemeral nature. However, short-term effects of NO on β-cell functioning have not been assessed, previous studies employing long incubation times and sampling intervals.

In order to establish a more precise role for NO in β-cell functioning, this study investigated short-term effects of aqueous NO on the $[Ca^{2+}]_i$ and secretory activity of isolated rat pancreatic β-cells. Analysis of β-cell secretory activity was performed by measuring the secretion of 5-hydroxytryptamine (5-HT) from pre-loaded cells, using a carbon fibre electrode. This species is taken up by β-cells and is localised to the secretory granules [9]. It is co-secreted along with insulin in response to a variety of secretagogues [10]. We have therefore exploited the ability of β-cells to take up, store and release 5-HT as a means of detecting the release of secretory granule contents. In this study we found that NO both mobilized intracellular Ca^{2+} and stimulated secretory activity of isolated β-cells, over a similar time-course. Our findings support a role for NO as a signalling molecule in β-cell insulin secretion.

2. Materials and methods

2.1. Preparation of cells

Following removal of pancreata from 200–400 g Wistar rats, islets were prepared by collagenase digestion as previously described [11]. Isolated cells and small clusters were obtained by dissociating the islets in a Ca^{2+} free trypsin/EDTA solution. Cells were then plated onto glass cover-slips and maintained in RPMI 1640 medium supplemented with 11 mM glucose, 10% foetal calf serum (Gibco), 10 μg/ml streptomycin (Gibco), and 10 μg/ml penicillin (Gibco). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and were maintained in culture up to 3 days prior to use.

2.2. Analysis of free intracellular Ca^{2+} by single wavelength microfluorimetry and video ratio imaging

Microfluorimetric analysis of β-cell $[Ca^{2+}]_i$ was performed using standard fluorescence microscopy, in a similar way as previously described [11]. Cells were loaded with Ca^{2+} indicator by incubation with 1 μM of the acetoxymethyl (AM) ester of Fluo-3 (Molecular Probes Inc.) in modified Hank's medium containing 2.8 mM glucose, at 22–24°C for 30 min. This glucose concentration was sub-threshold for increasing the $[Ca^{2+}]_i$ of isolated rat β-cells in our experiments. Cells were incubated for a further 30 min before recordings were made, to

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allow completion of ester hydrolysis. Fluo-3 fluroescence was excited at 485 nm and the emission intensity measured at 530 nm. Experiments were carried out at 32–34°C, with cells maintained in modified Hank's medium. Fluorescence intensity was not calibrated, data being presented only as the relative change in signal.

Video ratio image analysis of β-cell $[Ca^{2+}]_i$ was performed using an ion imaging system supplied by 'Improvision', Coventry, UK, in a similar way as previously described [12]. Cells were loaded with the acetoxymethyl (AM) ester of Fura-2 (Molecular Probes Inc.), by the same method as for Fluo-3 AM. Free cytosolic Ca^{2+} concentration was determined by taking the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm, using an emission wavelenghth of 510 nm. Pairs of 340 and 380 nm images were captured every 8 s and ratio images were calculated using 'Ionvision' software ('Improvision', Coventry, UK). Experiments were performed at 22°C, with cells maintained in modified Hank's medium. Standard $CaCl_2$ solutions were used to calibrate the system, and viscosity corrections were made [12].

2.3. Measurement of 5-hydroxytryptamine secretion in β-cells

Analysis of β-cell secretory activity was carried out by measuring the secretion of 5-hydroxytryptamine (5-HT) from pre-loaded cells using a carbon fibre electrode, as previously described [13]. Cells were pre-loaded with the amine by overnight incubation in tissue culture medium supplemented with 0.5 mM 5-HT and 0.5 mM of the 5-HT precursor, 5-hydroxytryptophan. Subsequent secretion of 5-HT from a loaded cell was detected with a carbon fibre electrode placed next to it. The electrode was held at 600 mV, and secretion was monitored by measuring the current associated with oxidation of the vesicle contents. All secretion experiments were performed at 32–34°C in modified Hank's medium supplemented with 5 μM forskolin.

2.4. Drugs and solutions

NO solution was prepared by the following method; a degassed 3 ml aliquot of modified Hank's medium was transferred to an air-tight bottle, and was bubbled with nitrogen for 10 min at 4°C. This solution

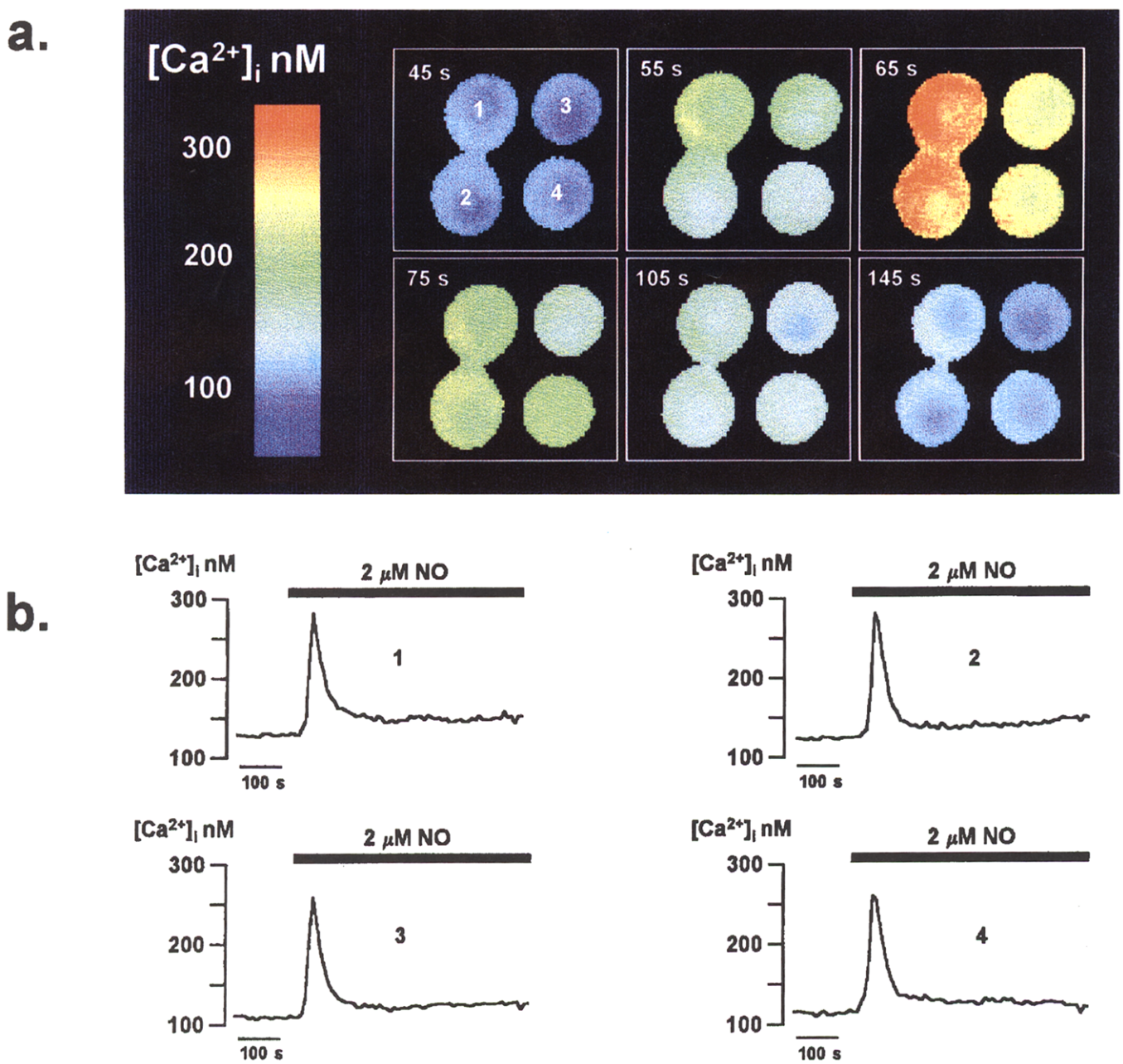

Fig. 1. NO-induced transient increases in $[Ca^{2+}]_i$ of rat pancreatic β-cells, measured by video ratio imaging. (a) Consecutive pseudocolour ratio-images of 4 β-cells loaded with Fura-2, after treatment with 2 μM NO at times indicated. (b) Individual traces of $[Ca^{2+}]_i$ vs. time for the cells shown in (a). Data are representative of responses from at least 40 cells obtained from 7 different experiments.

was then bubbled with NO (Aldrich) for 10 min at 4°C, yielding a saturated solution of approximately 1 mM NO [14]. NO-solution was stored air-tight, at 4°C for up to 1 day. Experiments were performed in modified Hank's medium (pH 7.2), containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.83 mM $MgSO_4$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 2.8 mM glucose. For experiments carried out in low Ca^{2+} medium, $CaCl_2$ was ommitted from the above, and 3 mM EGTA was added, yielding a free Ca^{2+} concentration of ~1 nM. All drugs were from Sigma.

3. Results

In these and the following experiments, β-cells were firstly identified by the addition of 10 mM glucose to the incubation bath. This treatment raises the $[Ca^{2+}]_i$ of β-cells. Following β-cell identification, the incubation medium was replaced with Hank's medium containing 2.8 mM glucose, and $[Ca^{2+}]_i$ was monitored until it stabilised at the prestimulatory level, prior to commencement of the experimental protocol.

Addition of 2 μM NO solution to the incubation bath resulted in a homogeneous and transient increase in the $[Ca^{2+}]_i$ of β-cells, as assessed by video ratio imaging (Fig. 1). With a normal extracellular Ca^{2+} concentration of 1.3 mM, the amplitude of the response was typically 150–200 nM above the basal Ca^{2+} level (~120 nM) of isolated cells, with response duration of approximately 100 s (Fig. 1). In order to determine whether the NO-induced response was dependent on extracellular Ca^{2+}, cells were bathed for at least 3 min in Hank's medium where Ca^{2+} had been chelated, prior to the addition of NO. Transfer of β-cells into this medium (0 Ca^{2+}, 3 mM EGTA) caused a lowering of their basal $[Ca^{2+}]_i$ from approximately 120 to 60 nM (Fig. 2a). Addition of 2 μM NO to these cells also resulted in an increase in $[Ca^{2+}]_i$ (Fig. 2a), indicating that the response was partly, or wholly due to release of Ca^{2+} from intracellular stores. In this medium, response amplitudes were reduced to approximately 100 nM above the basal Ca^{2+} level, while response duration was prolonged. A smaller response in this medium may either suggest that the NO response is comprised of a Ca^{2+} influx component, or that the NO-sensitive store had been partly depleted by prior incubation of the cells in low Ca^{2+} medium. The prolonged response duration suggests that Ca^{2+} resequestration and/or efflux may be Ca^{2+} dependent in these cells.

To determine whether the NO-induced Ca^{2+} mobilization of rat β-cells involved release of Ca^{2+} from endoplasmic reticulum (ER) stores, cells were pretreated with the ER Ca^{2+}-ATPase inhibitor, thapsigargin. Previous studies have shown thapsigargin to be effective in depleting non-mitochondrial Ca^{2+} pools of insulin secreting cell lines; interestingly, in these cells, the thapsigargin-sensitive Ca^{2+} pools were not entirely $Ins(1,4,5)P_3$ sensitive, an excess of $Ins(1,4,5)P_3$ being capable of releasing only a fraction of the Ca^{2+} in these pools [15].

Addition of 10 μM thapsigargin to isolated rat β-cells, main-

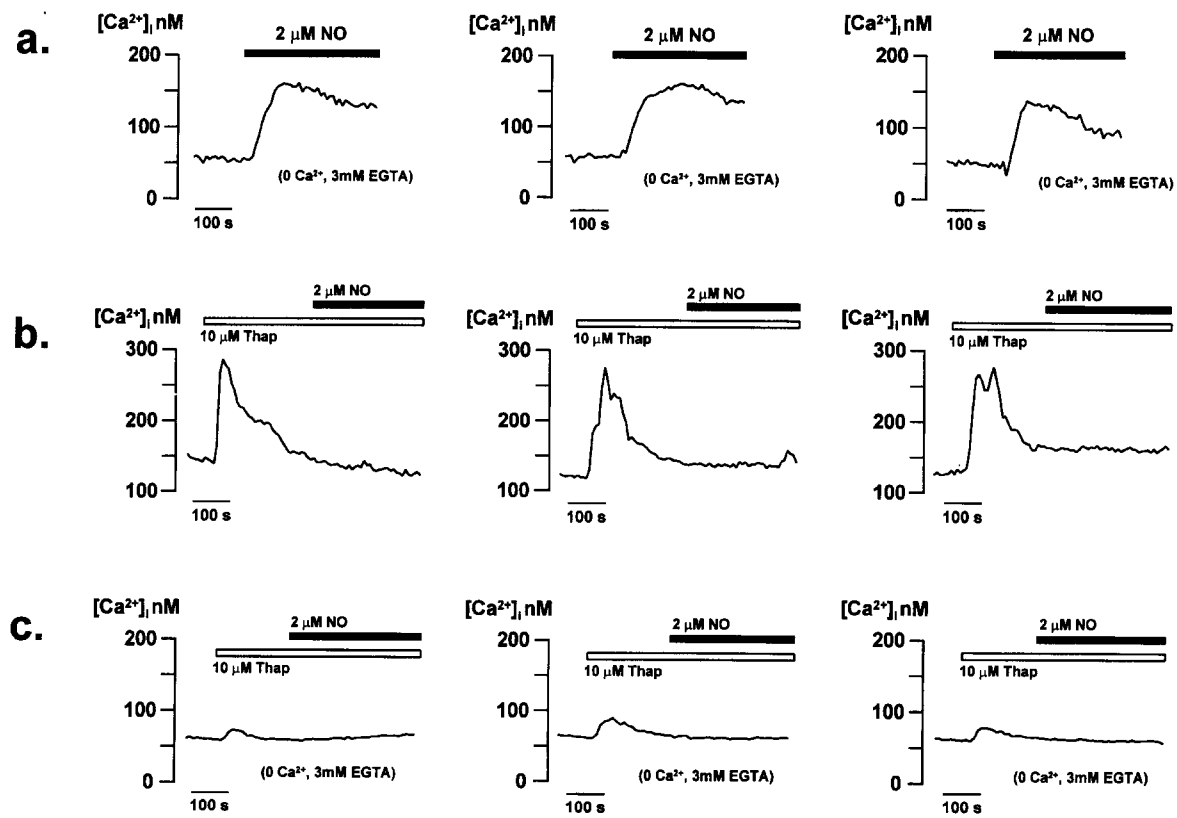

Fig. 2. NO-induced transient increases in $[Ca^{2+}]_i$ of β-cells maintained in low Ca^{2+} medium and inhibition of the NO-induced Ca^{2+} response by thapsigargin. (a) Traces of $[Ca^{2+}]_i$ vs. time derived from video ratio imaging of β-cells maintained in low Ca^{2+} (0 Ca^{2+}, 3 mM EGTA) medium, and treated with 2 μM NO. Data are representative of responses from at least 30 cells obtained from 5 different experiments. (b) traces of $[Ca^{2+}]_i$ vs. time for β-cells maintained in normal (1.3 mM), or low Ca^{2+} (0 Ca^{2+}, 3 mM EGTA) medium (c) and pretreated with 10 μM thapsigargin, prior to addition of 2 μM NO. In (b) and (c), data are representative of responses from at least 35 cells obtained from 6 different experiments.

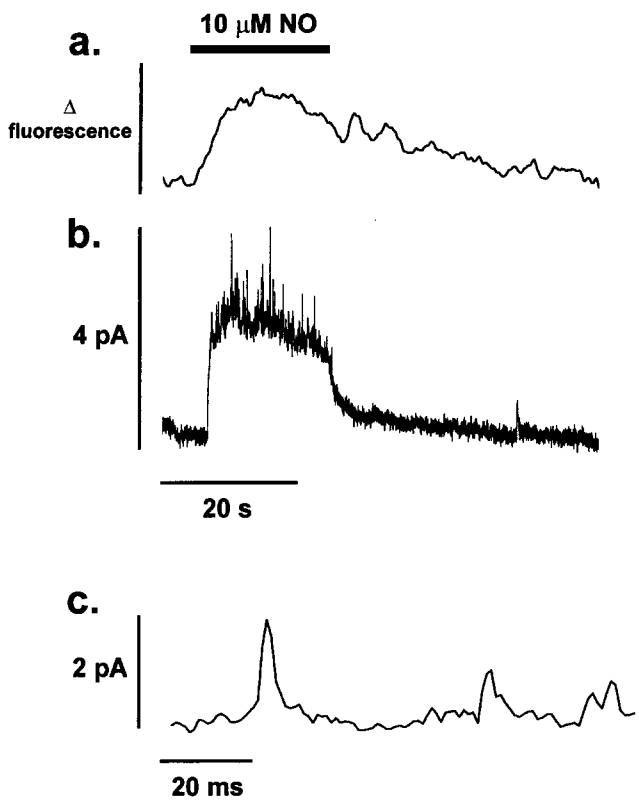

Fig. 3. NO-induced transient increase in β-cell $[Ca^{2+}]_i$ and 5-HT secretion, measured simultaneously at 32–34°C by amperometry, and single wavelength microfluorimetry, respectively. (a) Trace of relative change in Fluo-3 fluorescence vs. time, for a β-cell treated with a local puff of 10 μM NO solution. (b) Amperometric current record from the same cell as in (a), showing oxidation current spikes associated with the release of 5-HT. (c) Expansion of secretory current spikes in (b), supporting vesicular release of 5-HT. Data are representative of responses from at least 7 cells.

tained in normal (1.3 mM) or low Ca^{2+} (0 Ca^{2+}, 3 mM EGTA) medium, resulted in a transient increase in $[Ca^{2+}]_i$ (Fig. 2b,c). On recovery of the $[Ca^{2+}]_i$ to a new basal level, 2 μM NO was added to the bath. NO failed to elicit a response following this treatment (Fig. 2b,c), suggesting that the NO-sensitive Ca^{2+} store was entirely depleted by pretreatment with thapsigargin. The response to thapsigargin was considerably smaller for cells transferred to low Ca^{2+} medium, suggesting that this treatment alone partly depleted the thapsigargin-sensitive store, or that thapsigargin's efficacy is dependent on the $[Ca^{2+}]_i$.

Localised puff application of 10 μM NO to a single β-cell resulted in the release of 5-HT (Fig. 3b,c). A corresponding transient increase in the $[Ca^{2+}]_i$, was simultaneously measured by single wavelength microfluorimetry (Fig. 3a). Ca^{2+} response profiles were similar to those observed with bolus additions of NO and video ratio imaging of Fura-2 loaded cells (Fig. 1). With NO-free solution in the puffing pipette, Ca^{2+} or 5-HT secretory responses were not elicited. Fig. 3 demonstrates a close correlation for the onset and duration of both the increase in secretory activity (5-HT release) and increase in $[Ca^{2+}]_i$, consistent with $[Ca^{2+}]_i$ being functionally coupled to secretion in these cells. Similar Ca^{2+} and secretory responses (5-HT release) to NO were observed in β-cells maintained in low Ca^{2+} (0 Ca^{2+}, 1 mM EGTA) medium (data not shown). This suggests that the NO-induced secretory response (5-HT release) is predominantly coupled to Ca^{2+} mobilization and not to Ca^{2+} influx in these cells.

The intracellular Ca^{2+} release mechanism activated by NO was further investigated by determining whether this mechanism was ryanodine receptor (RyR)-linked. It is possible that rat β-cells possess a RyR-mediated Ca^{2+} pool, since the pharmacological effector of the RyR, caffeine, at a dose of 5–10 mM, induced transient increases in the $[Ca^{2+}]_i$ of these cells (data not shown). To test for the involvement of a RyR-mediated Ca^{2+} release mechanism, isolated cells were pretreated with antagonising concentrations of ryanodine. The binding of ryanodine to the RyR is practically irreversible, and at doses greater than 50 μM, the associated Ca^{2+} conductance is inhibited. Due to its slow association kinetics [16], ryanodine concentrations between 100–200 μM, and pre-incubation of up to 1 h were employed. These treatments did not increase the $[Ca^{2+}]_i$ of β-cells in our experiments.

The NO-induced Ca^{2+} responses of cells incubated with 100 μM ryanodine for 10 min, were significantly diminished (Fig. 4a). Response amplitudes were reduced to approximately 50% of control experiments (Fig. 1), suggesting that this concentration of ryanodine had partially inhibited the NO-induced response. Increasing the dose of ryanodine to 200 μM and the pre-incubation to 1 h, the response to NO was essentially eliminated (Fig. 4b). These data suggest that NO mobilizes Ca^{2+} via a ryanodine receptor-linked mechanism.

4. Discussion

This is the first study to demonstrate that low concentrations of aqueous NO induce both an increase in β-cell $[Ca^{2+}]_i$ and secretory activity (5-HT release). Taken as a whole, the data from this study suggest that NO mobilizes Ca^{2+} from a RyR-mediated Ca^{2+} pool, and that the resulting increase in $[Ca^{2+}]_i$ is coupled to an increase in the secretory activity (5-HT release) of rat β-cells. Several lines of evidence suggest the existence of a RyR-mediated Ca^{2+}-induced Ca^{2+}-release (CICR) pool in insulin secreting cells. From this study, the pharmacological activator of the RyR, caffeine, induced transient increases in the $[Ca^{2+}]_i$ of isolated rat β-cells, and the NO-induced Ca^{2+} response was blocked by preincubation with antagonising concentrations of ryanodine. From previous studies, the sulfhydryl reagent thimerosal, an activator of CICR, has been shown to release Ca^{2+} from an $Ins(1,4,5)P_3$-insensitive pool in the insulin secreting cell line, RINm5F [17], and in mouse pancreatic β-cells [18]. Also, it has been suggested that the putative physiological effector of the RyR, cyclic-ADP-ribose (cADPR) [19], not only mobilizes Ca^{2+}, but also stimulates insulin secretion, and is synthesised in response to D-glucose in rat β-cells [20]. Although the physical and functional nature of Ca^{2+} stores in β-cells are poorly defined, previous work suggests that $Ins(1,4,5)P_3$ sensitive and insensitive Ca^{2+} stores coexist in β-cells [15,17,18]. Therefore, it is possible that ryanodine and $Ins(1,4,5)P_3$ sensitive stores are distinct entities in these cells, and that interplay between these may be involved in both CICR, and oscillations in $[Ca^{2+}]_i$, as has been suggested in other cells [21].

In our study complete inhibition of the NO-induced reponse was not achieved by a 10 min preincubation of β-cells with 100 μM ryanodine but was achieved with a 1 h preincubation with

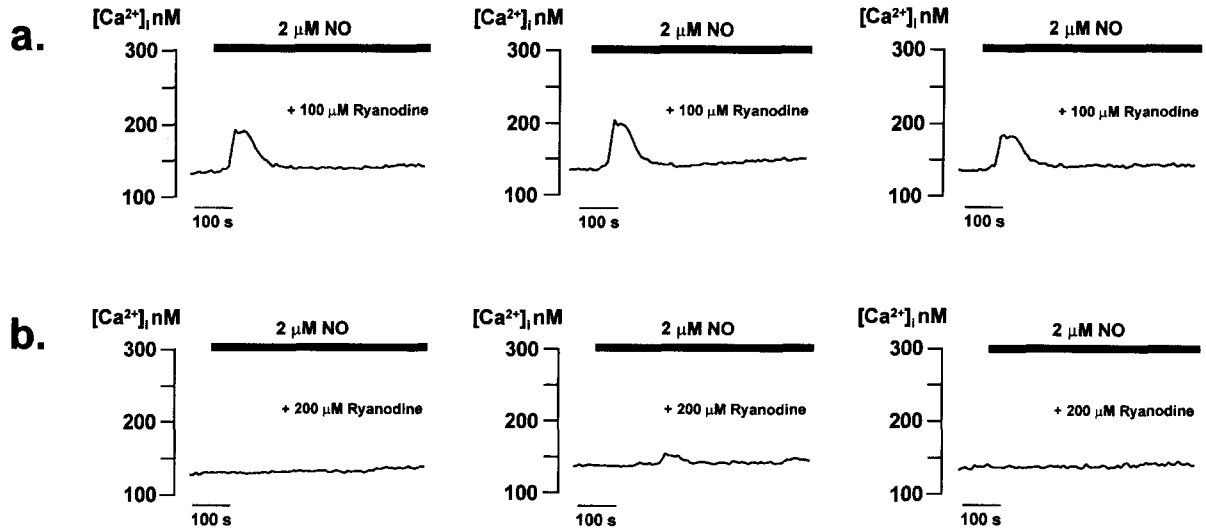

Fig. 4. Inhibition of the NO-induced Ca^{2+} response of β-cells by ryanodine. Traces of $[Ca^{2+}]_i$ vs. time for β-cells pretreated with either 100 μ M ryanodine for 10 min (a), or 200 μM ryanodine for 1 h (b), prior to addition of 2 μM NO. In (a) and (b), data are representative of responses from at least 40 cells obtained from 6 different experiments.

200 μM ryanodine. This probably reflects the binding kinetics of ryanodine to the RyR, where the half life for the association rate has been reported to be as slow as 23 min in muscle [16]. Inhibition of the NO effect by ryanodine appeared specific, and not due to a run-down phenomenon caused by prolonged incubation of cells, since control experiments showed that NO responses were retained following a 60 min incubation in normal solution.

Considering our data, and evidence that NO is generated in β-cells in response to D-glucose [3], it is tempting to speculate an apparent physiological role for NO in glucose-induced β-cell insulin secretion. Since NO synthesis is Ca^{2+} dependent in many cells possessing a constitutive NO-synthase [7], it is possible that glucose stimulates NO production in β-cells through an increase in $[Ca^{2+}]_i$; conventional K_{ATP} channel inhibition and subsequent activation of Ca^{2+} influx through L-type Ca^{2+} channels would be the likely mechanism for this [1]. Glucose-induced insulin secretion might therefore be the result of combined contributions of an increase in Ca^{2+} influx and NO-mediated Ca^{2+} mobilization in β-cells. This is difficult to test since glucose alone produces a heterogenous increase in $[Ca^{2+}]_i$, where latency, amplitude and duration of the responses vary; hence the results are likely to be ambiguous if NOS inhibitors were used in an attempt to further dissect this proposed mechanism. Furthermore, it is well established that arginine, in the presence of glucose, will stimulate secretion via cell depolarization, due to its transport across the plasma-membrane. Hence any involvement of arginine metabolism in the glucose-induced Ca^{2+} response would also be difficult to assess. Finally in vivo, NO may actually be supplied to the β-cells from the islet vasculature or neighbouring cells. Indeed, it has been demonstrated that a rise in blood glucose produces an increase in islet blood flow via NO [22].

The precise mechanism whereby NO mobilizes intracellular Ca^{2+} is yet to be established. From previous studies, it is possible that NO exerts its effect through the generation of cGMP; cGMP might in turn stimulate the production of the Ca^{2+} mobilizing agent, cADPR, via the activation of ADP-ribosyl-cyclase, as in the sea urchin egg [12]. Although the above mechanism seems attractive, recent studies have suggested that cADPR is ineffective in releasing intracellular Ca^{2+} from permeabilised rat pancreatic islet [15] or mouse β-cells [18], and from the insulin secreting cell lines INS-1 [15] or RINm5F [18]. Hence, whether cADPR is involved in Ca^{2+} mobilization and insulin secretion in β-cells remains controversial.

A previous study has demonstrated NO-induced Ca^{2+} mobilization in interstitial cells from canine colon [14]. This NO-induced Ca^{2+} mobilization was also inhibited by ryanodine pretreatment, suggesting a RyR-linked mechanism, as for the rat β-cells of this study. It will be interesting to see whether NO-induced Ca^{2+} mobilization is a wide ranging mechanism which is both functionally significant and common to other signal transducing systems. It will also be pertinent to determine whether NO generation within cells is coupled to cADPR production, and whether this species mediates the NO-induced Ca^{2+} mobilization witnessed in the above cell types.

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