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SEGREGATION OF NITRIC OXIDE SYNTHASE EXPRESSION AND CALCIUM RESPONSE TO NITRIC OXIDE IN ADRENERGIC AND NORADRENERGIC BOVINE CHROMAFFIN CELLS

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Abstract—Previous work has demonstrated that nitric oxide can be an important intracellular messenger in the regulation of neurosecretion in chromaffin cells. Since standard chromaffin cell cultures are mixed populations of noradrenaline and adrenaline producing cells, it would seem important to understand the functional differences between these individual components. The use of fluorescence imaging techniques for the recording of cytosolic calcium from single chromaffin cells together with the immunoidentification of individual cells with specific antibodies against tyrosine hydroxylase, *N*-phenyl ethanolamine methyl transferase and nitric oxide synthase, has allowed us to measure single-cell calcium responses in identified adrenergic, noradrenergic and nitrergic chromaffin cells, thus helping us to clarify the differential role of nitric oxide in the function of these chromaffin cell types. 53 ± 2% of chromaffin cells were able to synthesize nitric oxide (nitric oxidesynthase-positive cells), these cells being mainly noradrenergic (82 ± 2%). Results indicate that nitric oxide donors such as sodium nitroprusside, molsidomine and isosorbide dinitrate evoke $[Ca^{2+}]_i$ increases in a 62 ± 4% of chromaffin cells, the response to nitric oxide donors being between 30 and 50% of that of 20 μM nicotine. Cells responding to nitric oxide donors were mainly adrenergic (68 ± 5%) although 45 ± 9% of noradrenergic cells also gave $[Ca^{2+}]_i$ increasing responses. The distribution of nitric oxide responding cells between nitric oxide synthase-positive and negative was very similar in the whole population (63 ± 5 and 60 ± 7%, respectively), but these differences were more prominent when considering the distribution of nitric oxide response between noradrenergic and adrenergic nitric oxide synthase-positive cells; while 73 ± 6% of adrenergic nitric oxide synthase-positive cells evoke $[Ca^{2+}]_i$ increases by nitric oxide stimulation, only 35 ± 11% of noradrenergic nitric oxide synthase-positive cells respond.

Taken together these results seem to indicate that (i) nitric oxide could act within adrenal medulla as both an intracellular and intercellular messenger; and (ii) noradrenergic cells seem to be specialized in nitric oxide synthesis while adrenergic cells with an endocrine function could mainly act as a target of neurosecretory action of this second messenger. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: nitric oxide, chromaffin cells, catecholamines, calcium, immunocytochemistry.

Nitric oxide (NO) is a short-lived, highly reactive radical, originally identified as both a mediator in vasodilatation[22] and an active agent in macrophage cytotoxicity.[25] In addition NO has been shown to function as a central and peripheral neuronal messenger or putative neurotransmitter involved in crucial physiological events such as neurotransmitter release,[13] long-term potentiation[12] and gene transcription,[11,31] as well as in the pathophysiological events underlying neurotoxicity.[2,37]

In central and peripheral neurons NO is synthesized from L-arginine by the neural isoform of nitric oxide synthase (nNOS), which is structurally, immunologically and functionally different from the isoforms expressed constitutively in vascular endothelial cells or upon induction in macrophages.[18] The presence of this enzyme has been shown in different peripheral neural tissues by both biochemical analysis and immunofluorescence techniques.[1] In

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Abbreviations: AMCA, 7-amino-4-methyl-coumarin-3-acetic acid; BSA, bovine serum albumin; $[Ca^{2+}]i$, intracellular free calcium concentration; ChAT, choline acetyltransferase; DABCO, diazabicyclo[2,2,2]octane; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; ISDN, isosorbide dinitrate; nNOS, neural isoform of nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PNMT, *N*-phenyl ethanolamine *N*-methyl transferase; SNP, sodium nitroprusside; TH, tyrosine hydroxylase; TRITC, tetramethylrhodamine isothiocyanate.

these tissues NO plays its physiological functions by activation of a soluble guanylate cyclase resulting in formation of cGMP.

In the adrenal medulla, a tissue of ectodermic origin (as are neurons), the existence of a complete pathway L-arginine/NO/cGMP has been demonstrated. The presence of NOS has been shown in bovine chromaffin cells by both biochemical methods demonstrating the formation of L-citrulline from L-arginine[27,30] and by immunohistochemical methods using antisera raised against purified soluble nNOS[3] or cytochemical techniques showing a specific staining for NADPH-diaphorase.[27] By using these techniques its presence has been also assessed in adult rat adrenal gland in almost all choline acetyltransferase (ChAT)-positive fibres and ChAT/enkephalin preganglionic fibres running in the splanchnic nerves[16] and in small intra-adrenal non-cholinergic/non-adrenergic ganglion neurons which express vasoactive intestinal polypeptide-like immunoreactivity that are scattered between chromaffin cells.[9] In human adrenal medulla, immunoreactivity for NOS has also been demonstrated in neurons, nerve fibres and chromaffin cells, co-localizing with substance P only and not with neuropeptide Y and somatostatin.[15] The location of NOS in distinct structural compartments of the rat and human adrenal medulla indicates that NO is produced in different cell types and may reflect a differential role of this messenger system in autonomic control of adrenal gland function.

Concerning the role of NO in the regulation of adrenal medulla function previous work indicates that nitrergic compounds[7] or pure NO[27] activate basal and inhibit nicotine-evoked catecholamine secretion. However, while there is general agreement with the fact that NO pure gas,[27] NO-generating compounds and cGMP[29,32,33] decrease both catecholamine secretion and intracellular free calcium concentration ($[Ca^{2+}]_i$) increases evoked by depolarizing stimuli like acetylcholine, nicotine and high KCl, there are contradictory results concerning the effect of NO on basal catecholamine secretion and $[Ca^{2+}]_i$ since some authors failed to find any effect of NO on catecholamine secretion by itself.[24,29,32,33] In disagreement with these positive observations, Marley *et al.*[20] failed to find specific staining for NADPH-diaphorase and nNOS in adrenal medullary chromaffin cells and nerve fibres or any effect of NO in adrenaline and noradrenaline secretion evoked by electric field stimulation, concluding that NO does not play a direct role in the acute regulation of adrenal catecholamine secretion.

All these results show that the effect of NO on the function of chromaffin cells is more complex than it previously seemed and it is far from being clear. A major source of uncertainty that may explain, at least in part, these conflicting results, may be the known cellular heterogeneity of bovine adrenal medulla and chromaffin cell cultures.[10,23] Functionally different responses to histamine and angiotensin II,[5,26] ATP[4] and Ca^{2+},[35] were demonstrated among adrenergic and noradrenergic or ganglionar cells co-existing in these mixed cultures. So, given this heterogeneity it seems very important to investigate the possible functional differences between these cellular types with respect to NO action. For example, it is important to know if NO-evoked responses are limited to a unique population of noradrenergic or adrenergic chromaffin cells and if they correspond to NO-producing (NOS^+) or non-producing (NOS^-) chromaffin cells.

Since a key messenger in the neurosecretory function of chromaffin cells is Ca^{2+}, whose signalling pathways are deeply involved in these as well as many other neuronal processes,[21] we have combined the fluorescence imaging techniques for the recording of cytosolic free calcium from single chromaffin cells with the immunoidentification of individual cells using specific antibodies against tyrosine hydroxylase (TH), phenyl ethanolamine *N*-methyl transferase (PNMT) and nNOS, in order to be able to ascribe single cell calcium responses to identified chromaffin cells. It has helped us to clarify the differential role of NO in the function of adrenaline and noradrenaline secreting cells as well as in NO-producing cells.

EXPERIMENTAL PROCEDURES

Cell culture

Bovine chromaffin cells were obtained essentially in the same way as described previously.[8,17] Briefly, after digestion by retrograde perfusion with collagenase the medullary tissue was collected, disgregated and chromaffin cells purified in a Percoll density gradient. The cells were plated onto 16 mm round coverslips coated with poly-L-lysine. Chromaffin cells were maintained under a 5% CO_2/95% air humidified atmosphere at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with 15 mM HEPES, 25 mM $NaHCO_3$, 5% inactivated foetal calf serum, penicillin and streptomycin. Cells were typically used between days 2 and 6 after plating.

Microfluorescence set-up

The $[Ca^{2+}]_i$ was measured with the fluorescent probe Fura-2. The coverslips containing the cells were washed in physiological saline containing (in mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose. The solution was constantly gassed with 95% O_2/5% CO_2 for a final pH of 7.4. Chromaffin cells were loaded with 2.5 μM Fura-2/AM for 45 min at 37°C in this medium supplemented with 1% bovine serum albumin (BSA). After washing, the coverslip was glued to the bottom of a small (approx. 100 μl) perifusion chamber and placed in the stage of a Nikon Diaphot TMD microscope. The cells were continuously perifused (approx. 1.5 ml/min) with gassed physiological saline. The drugs were applied for short periods (15–60 s) dissolved in the perifusion medium with the aid of a four-way stopcock.

Measurement of $[Ca^{2+}]_i$ by video imaging

The fluorescence changes were recorded with a multiple excitation MagiCal imaging system (Applied Imaging, U.K.). Chromaffin cells were alternately excited at 340 nm and 380 nm by means of a stepping filter wheel and the epifluorescence optics of the microscope. Emitted fluor-

escence collected with a 20× objective was driven to a Photonics Science SIT camera after passing through a 510 nm bandpass filter. Eight frames (approx. 100 ms exposure) were averaged to produce each image. Alternative excitation, image capture and processing were controlled by a single processor in the MagiCal system. Image analysis was performed with MagiCal software and custom-made programs (developed by E.C., details available on request). Essentially, background fluorescence at each wavelength (obtained from a field devoid of cells in each coverslip) was subtracted and fluorescence images ratioed on a pixel-by-pixel basis. Ratio data were stored as 8-bit pseudocoloured images. A contour was drawn around each cell in a field and the averaged ratio value of pixels inside each contour evaluated at each time point, in order to obtain ratio vs time plots for all cells.

Immunocytochemical identification of chromaffin cells

To study the distribution of NOS, the cells were double immunostained with antibodies against NOS (mouse monoclonal anti-NOS) and either TH or PNMT (rabbit polyclonals anti-TH and anti-PNMT). Briefly, cells were fixed for 2 min in ice-cold 1:1 acetone:methanol mixture. After blocking in phosphate-buffered saline (PBS) with 3% BSA/0.1% Triton X-100, the preparation was incubated with primary antibodies (mouse anti-NOS 1:100, rabbit anti-TH or anti-PNMT 1:500) for 1 h at room temperature. After washing in 0.1% Triton X-100, secondary antibodies were added [fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG and tetramethylrhodamine isothiocyanate (TRITC)-labelled goat anti-rabbit IgG at 1:200] and incubated for another hour.

To identify the cells responding to NO donors, triple labellings with mouse anti-NOS, rabbit anti-PNMT and mouse anti-TH (monoclonal) were performed in the same coverslips used for $[Ca^{2+}]_i$ measurements. Once the $[Ca^{2+}]_i$ experiments had been performed the cells were fixed with 50% acetone:50% methanol (v/v) for 1–2 min at 4°C, followed by freezing and storage at −20°C until processing. For the immunofluorescence assay, the fixed coverslips were thawed by placing them in ice-cold 50% acetone:50% methanol and warmed until at room temperature; all the following procedures were performed at this temperature. The preparation was first processed as for double labelling using anti-NOS and anti-PNMT primary antibodies and FITC- and TRITC-secondary antibodies. After washing, the preparation was fixed again in 1:1 acetone:methanol in order to denaturalize the already bound mouse anti-NOS primary antibody[14] and processed for labelling with mouse anti-TH (1:50) and 7-amino-4-methyl-coumarin-3-acetic acid (AMCA)-conjugated anti-mouse IgG (1:100). The coverslips were mounted in 50% glycerol in PBS containing 2.5% diazabicyclo[2,2,2]octane (DABCO) and digital images taken with the same MagiCal system used for Ca^{2+} experiments.

Statistical analysis

Fluorimetric data are expressed as mean ± S.E.M. net ratio increases. Statistical comparisons between means were performed with a Student's *t*-test. Immunocytochemical data are expressed as number of cells counted and as proportion ± S.D. of labelled cells vs total cells. S.Ds were calculated assuming a binomial distribution of counted cells. Comparisons between proportions in 2 × 2 tables were done with a χ^2 test.[19] Table 3 was analysed with a bivariate ANOVA approach followed by χ^2 tests of individual rows.[34]

Materials

Fura2/AM was from Molecular Probes (Eugene, OR, U.S.A.) and collagenase was from Boehringer Mannheim S.A. (Barcelona, Spain). DMEM and fetal calf serum were purchased from GIBCO (BRL, U.K.). Antibiotics were supplied by Flow Laboratories Ltd (Irvine, CA, U.S.A.). Sodium nitroprusside (SNP), isosorbide dinitrate (ISDN), molsidomine, BSA and anti-IgG-FITC and TRITC secondary antibodies were obtained from Sigma Chemical (Madrid, Spain). Mouse monoclonal anti-nNOS IgG was from Affinity Research Products Ltd (Nottingham, U.K.). Rabbit polyclonals anti-TH and rabbit anti-PNMT were from Eugene Technology International, mouse monoclonal anti-TH was from Boehringer Mannheim S.A. and AMCA-conjugated anti-mouse IgG from Calbiochem (San Diego, CA, U.S.A.). All other reagents were from Merck (Darmstadt, Germany).

RESULTS

Nitric oxide donors evoke $[Ca^{2+}]_i$ rises in a subpopulation of chromaffin cells

We observed rapid NO-donor induced changes in $[Ca^{2+}]_i$ in individual chromaffin cells (Fig. 1) with the aid of digital imaging fluorescence microscopy. The stimulation with these NO-donors produced different types of time courses, sometimes mono- and other times multi-phasic peaks. Representative time courses of changes in $[Ca^{2+}]_i$ in a few cells are shown in Fig. 1. The most common pattern of response encountered after 100 μM molsidomine stimulation was a moderately increasing signal reaching a plateau (Fig. 1A). In contrast with 100 μM SNP and ISDN the most common pattern of response was a transient (mono or biphasic) peak returning to basal levels after removing the compound (Fig. 1B, C). The resting $[Ca^{2+}]_i$ was about 197 ± 43 nM in normal medium containing 2.5 mM $CaCl_2$. Stimulation of cells with 100 μM molsidomine, SNP or ISDN (the dose that doubled NO_2^- production levels), resulted in a net increase in the Fura-2 F_{340}/F_{380} ratio of between 0.25 and 1.25 (about 50 and 450 nM), the more efficient agonist being molsidomine (Fig. 1A). The time course of calcium responses was different after challenging cells with 20 μM nicotine. In this case, all the cells displayed rapidly increasing ratio transients that gradually decayed after washing the agonist and with peak heights larger than those obtained with NO-donors. The calcium increases in the case of NO-donors represented between 30 and 50% of the $[Ca^{2+}]_i$ rises obtained with 20 μM nicotine in the same cells.

The frequency histograms showing the distribution of the three NO-donor increases in Fura-2 ratios (Δ ratio) in the whole chromaffin cell population are shown in Fig. 2. From the total chromaffin cell population the 57% responded to 100 μM molsidomine, 36% to 100 μM SNP and 37% to 100 μM ISDN (Fig. 2). The maximal average ratio increase corresponded to molsidomine (0.52 ± 0.08, mean ± S.E.M.), followed by SNP (0.45 ± 0.04) and ISDN (0.4 ± 0.05), respectively. These parameters were collected from nine different experiments on a total of 125 chromaffin cells, among which 78 responded to NO-donors (62 ± 4%).

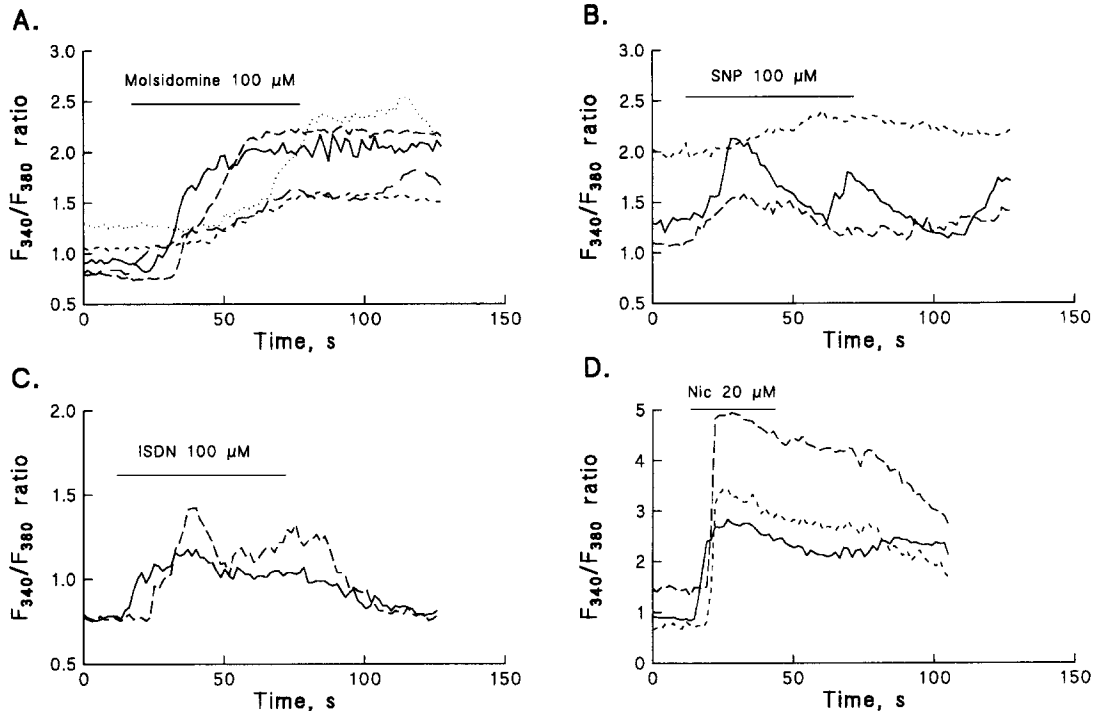

Fig. 1. Time courses of changes in $[Ca^{2+}]_i$ in individual chromaffin cells in response to stimulation with different NO-donors. Cells placed in a perifusion chamber in the stage of the microscope were imaged through a 20× fluor objective. The cells were stimulated with (A) 100 μM molsidomine, (B) 100 μM SNP and (C) 100 μM ISDN for a 60 s period. Pairs of images of fields were captured at 1.5 s intervals and the F_{340}/F_{380} ratio for each cell in the field was determined on a pixel by pixel basis as described in Experimental Procedures. Traces show representative examples of typical F_{340}/F_{380} ratio responses of individual cells and correspond to different fields during different experiments. The lines show the application period of the different NO-donors.

Nitric oxide synthase synthesizing cells can be identified by nitric oxide synthase immunocytochemistry

The immunoidentification of chromaffin cells was carried out by treatment of fixed chromaffin cells with different specific antibodies against TH, the rate-limiting enzyme for catecholamine biosynthesis (which stains the total chromaffin cell population), PNMT, the regulatory enzyme of adrenaline biosynthesis (marker of adrenergic population of chromaffin cells) and for nNOS, enzyme responsible for NO biosynthesis and marker of nitrergic cells. Noradrenergic cells were those stained by anti-TH but not by anti-PNMT antibodies.

Figure 3 shows the immunofluorescence images of chromaffin cells identified by double or triple immunostaining, in a total of 971 cells, with anti-nNOS and anti-TH antibodies (Fig. 3A, representative of 450 cells), anti-nNOS and anti-PNMT antibodies (Fig. 3B, representative of 396 cells) and anti-TH, anti-nNOS and anti-PNMT antibodies (Fig. 3C, representative of 125 cells).

By combining all the data from double and triple immunostainings we obtained the relative proportions of NOS^+ cells among adrenergic and noradrenergic populations shown in Table 1. NOS^+ cells comprise 53 ± 2% of the total population (Table 1A). NOS was preferentially expressed by noradrenergic cells (82 ± 2% NOS^+), while adrenergic cells show a much lower expression of this enzyme (26 ± 3% NOS^+) (Table 1B). The relative proportions of adrenergic and noradrenergic cells in total chromaffin cell population were 50 ± 2% in both cases (Table 1B). This proportion was quite different in NOS^- and NOS^+ cells; whereas in NOS^- cells there was a greater proportion of adrenergic (81 ± 2.5%, against 19 ± 2.5%), most of NOS^+ cells (76 ± 2.5%) were noradrenergic (Table 1B).

Distribution of chromaffin cell response to nitric oxide-donors in different chromaffin cell populations

Once we had the possibility of immunoidentifying the type of chromaffin cell as either adrenergic/noradrenergic or nitrergic/non-nitrergic we were able to assign single-cell calcium responses to identified chromaffin cells.

Table 2 shows the relative proportion of NO-donor responsive cells among adrenergic and noradrenergic chromaffin cells. From a total of 125 immunoidentified chromaffin cells in which Fura-2 ratio was measured, only a 62 ± 4% of the total population showed increases in Fura-2 ratio (Table

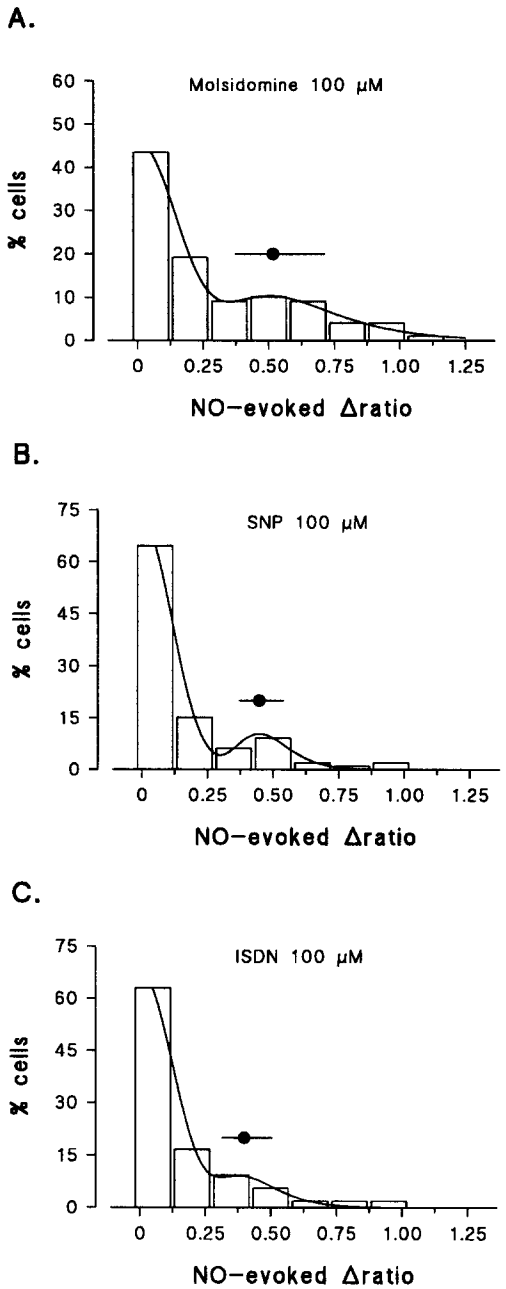

Fig. 2. Distribution of chromaffin cell Fura-2 calcium response to NO-donors in the whole chromaffin cell population. Frequency histograms show the distribution of increases in Fura-2 ratio (Δ ratio) evoked by (A) 100 μM molsidomine, (B) 100 μM SNP and (C) 100 μM ISDN measured as indicated in Experimental Procedures. Data are results from the analysis of 125 cells of which 62 ± 4% showed clear responses to NO-donors. First bars represent those cells unresponsive to NO donors. A log-normal distribution was fitted to the histogram representing responding cells. The average Δ ratio and ± 2 S.D. confidence limits for mean increase are shown by the point and lines above the histograms.

2A). This proportion of NO-donor responsive cells was quite similar in adrenergic cells (68 ± 5%) while in noradrenergic cells the proportion was significantly reduced, only 45 ± 9% of cells displayed increases in Fura-2 ratios (Table 2B).

Figure 4A, B shows the frequency histograms reflecting the distribution of 100 μM molsidomine-evoked ratio increase responses in adrenergic and noradrenergic chromaffin cells. Over 60% of adrenergic cells produced positive calcium responses, while only 45% of noradrenergic cells displayed molsidomine-evoked calcium responses. However, the average increase in the ratio evoked by 100 μM molsidomine was very similar in both chromaffin cell populations (0.54 ± 0.07 in adrenergic and 0.57 ± 0.12 in noradrenergic cells). Similar results were obtained when SNP and ISDN were used as NO-donors (data not shown). NO-responsive cells were not differentially segregated to NOS^+ and NOS^- chromaffin cells. The proportions of NO-responding cells were 63 ± 5% and 60 ± 7% for NOS^+ and NOS^- cells, respectively (Table 2C). However, the averaged ratio increases elicited by molsidomine in NOS^+ and NOS^- cells were significantly higher in NOS^- cells (0.48 ± 0.07 and 0.65 ± 0.06, respectively; Fig. 5A, B).

The results of analysing all the data for the three NO-donors show the relative proportions of NO-donor-responsive cells among NOS^+ and NOS^- and between adrenergic and noradrenergic chromaffin cells to be that indicated in Table 3. No significant difference exists between the adrenergic (59 ± 8%), noradrenergic (67 ± 16%) and total population (60 ± 7%) of NOS^- chromaffin cells while the relative proportion of NO-donor-responsive cells was significantly smaller in noradrenergic population of NOS^+ chromaffin cells (35 ± 11% against 73 ± 6% in noradrenergic and adrenergic cells, respectively).

DISCUSSION

Previous studies on the effects of NO in chromaffin or PC-12 cells have focused mainly on the effects on secretagogue-evoked catecholamine secretion or $[Ca^{2+}]_i$ increases.[6,32,33,36] Less attention has been paid to the direct action of NO on basal catecholamine secretion and $[Ca^{2+}]_i$, an effect in which the NO may be considered more as a primary neurotransmitter than a neuromodulator. This point appears to be somewhat controversial. Dohi *et al.*,[7] working on perfused dog adrenals, described a stimulatory effect of nitro-compounds, including NO, in catecholamine secretion, but this enhancement of catecholamine release was not observed in perfused cat adrenal medulla[24] nor in bovine cultured chromaffin cells.[29] Here we have demonstrated that NO-donors elicit clear $[Ca^{2+}]_i$-increasing responses in bovine chromaffin cells in culture. Since Ca^{2+} is the direct trigger for catecholamine release, this result supports our previous finding that NO gas can elevate basal catecholamine secretion.[27] The $[Ca^{2+}]_i$ increase elicited by NO donors was modest when compared with the responses to nicotine, but in

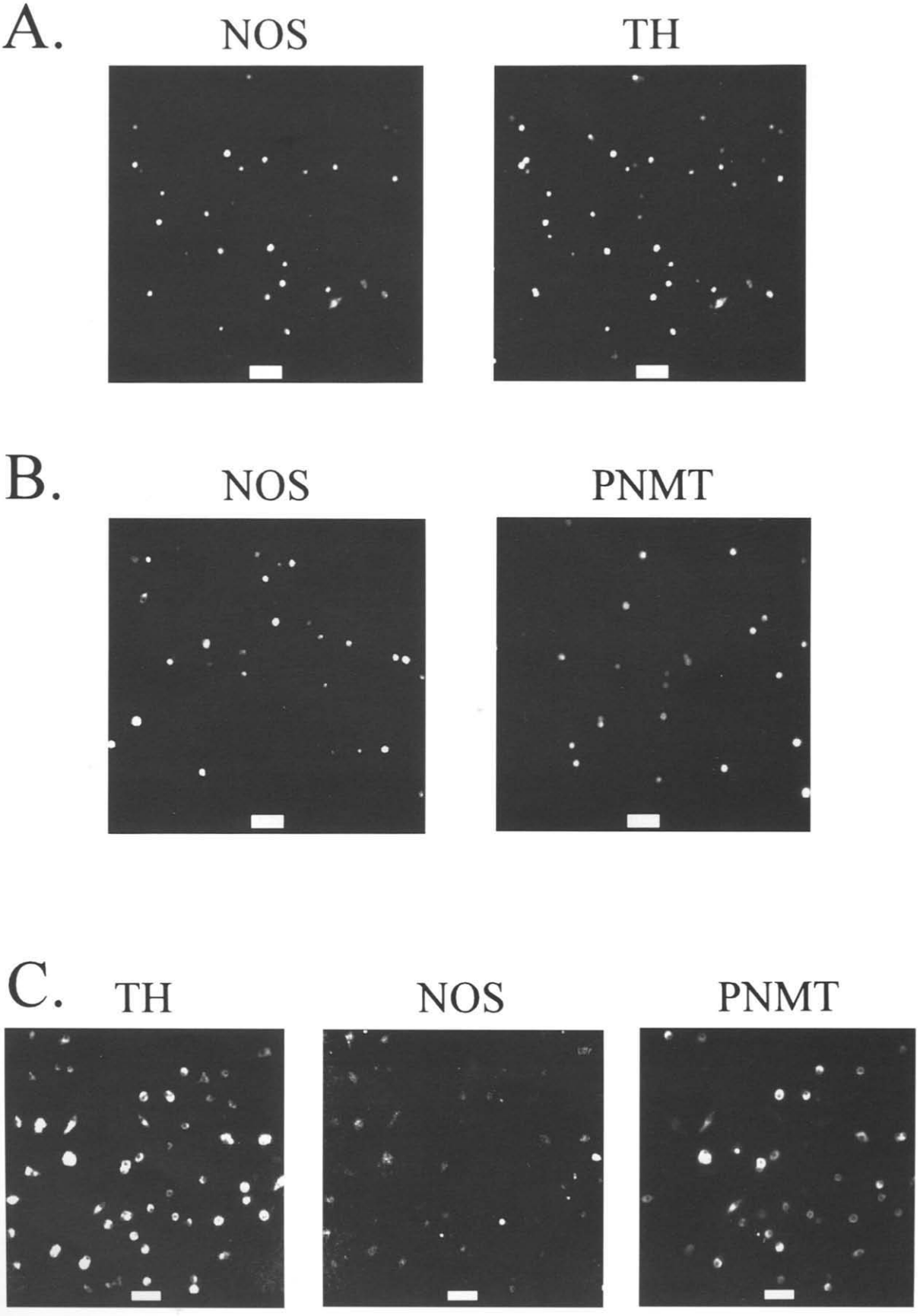

Fig. 3.

Table 1. Relative proportion of nitric oxide synthase-positive and negative cells in the whole chromaffin cell population (A) and within adrenergic and noradrenergic subpopulations (B)

	NOS^+		NOS^-		Total	
	n	%	*n*	%	*n*	
(A) Whole population	512	53±2	459	47±2	971	
(B) Adrenergic cells	69	26±3(13)	193	74±3(37)	262(50±2)	$P<0.001$
Noradrenergic cells	213	82±2(42)	46	18±2 (9)	259(50±2)	$P<0.001$
(total)	282	54±2	239	46±2	521	($P<0.001$)

The values within parenthesis are percentages calculated from the whole population. Data combining results from the three approaches indicated in Fig. 3. Collected from seven separate experiments and counting 22 independent microscopic fields. Statistical analysis: ± values were calculated as S.D. for a binomial distribution with the observed proportions. *P* values to the right represent testing the individual binomial proportion in each line against the 53% proportion observed in A. *P* value at the bottom right (in brackets) is the result of a χ^2-test for equal distribution of NOS in adrenergic and noradrenergic cells.

Table 2. Relative proportion of chromaffin cells responding to nitric oxide donors with a $[Ca^{2+}]_i$ increase in: (A) whole population; (B) adrenergic and noradrenergic cells and (C) nitric oxide synthase-positive and negative subpopulations of chromaffin cells

	$[Ca^{2+}]_i$-responsive		$[Ca^{2+}]_i$-unresponsive			
	n	%	*n*	%	total	
(A) Whole population	78	62±4	47	38±4	125	
(B) Adrenergic cells	65	68±5	31	32±5	96	
						$P<0.05$
Noradrenergic cells	13	45±9	16	55±9	29	
(C) NOS^+ cells	52	63±5	30	37±5	82	
						N.S.
NOS^- cells	26	60±7	17	40±7	43	

Data collected from nine different stimulations in three separate experiments. The observed difference between adrenergic and noradrenergic cells was significant as judged from a χ^2-test. N.S., non-significant.

proportion with the relatively smaller effect of NO on catecholamine secretion. In agreement with this view, the treatment of chromaffin cells with an analogue of cGMP, the proposed second messenger for NO actions, elicited a small increase in $[Ca^{2+}]_i$.[32] By using Fura-2 imaging techniques we have been able to reveal the heterogeneity of single cell responses to NO donors. Not all cells in a given culture displayed $[Ca^{2+}]_i$ rises elicited by NO-donors. Furthermore, the time course of $[Ca^{2+}]_i$ was highly variable, both in the onset and pattern of the response. Part of this variability may reside in the different kinetics of NO release by the NO-donors used. In fact, the response to nicotine, a direct agonist of acetylcholine receptors, occurred almost in synchrony in all cells. This heterogeneity, together with the fact that only a subpopulation (62±4%) of chromaffin cells responded to NO donors with $[Ca^{2+}]_i$ rises may explain why this effect has been elusive to others.

Bovine chromaffin cells in culture are a very popular model for the study of neurosecretion. In contrast to other species like guinea-pigs or chickens, bovine adrenal medulla contains both adrenergic and noradrenergic chromaffin cells. Although the adrenergic component predominates in the intact gland (around 70%), the proportion of adrenergic and noradrenergic cells in cultures varies with the method used for cell dissociation and purification.[23] Several studies have indicated that the distribution of receptors for ATP,[4] histamine,[5,26] angiotensin II[26] or GABA[28] is differentially segregated to adrenergic or noradrenergic cells. Thus the question arises as

Fig. 3. Immunofluorescence images showing the identification of chromaffin cells by specific enzymatic antibodies. Chromaffin cells were identified by immunostaining with specific antibodies as indicated in Experimental Procedures. (A) Double immunostaining of chromaffin cells with antibodies anti-TH (total population) and anti-nNOS (nitrergic cells). (B) Double immunostaining of chromaffin cells with antibodies anti-PNMT (adrenergic cells) and anti-nNOS (nitrergic cells). (C) Triple immunostaining of chromaffin cells with antibodies anti-TH (total population), PNMT (adrenergic cells) and anti-nNOS (nitrergic cells). In B and C, noradrenergic cells are stained by anti-TH but not by anti-PNMT antibodies. The plates are representative of several experiments, amounting to a total of 971 analysed cells. Scale bars = 50 μm.

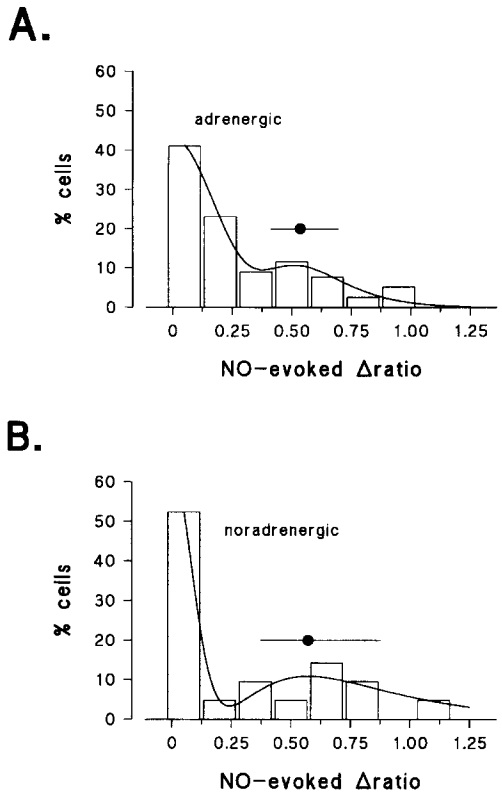

Fig. 4. Distribution of chromaffin cell response to NO-donors in whole, adrenergic and noradrenergic chromaffin cell populations. (A, B) Frequency histograms showing the distribution of 100 μM molsidomine evoked Δ ratio responses in adrenergic and noradrenergic chromaffin cells. The average increase in the ratio was very similar in both chromaffin cell populations. The response was not significantly higher in any of these subpopulations (Student's *t*-test).

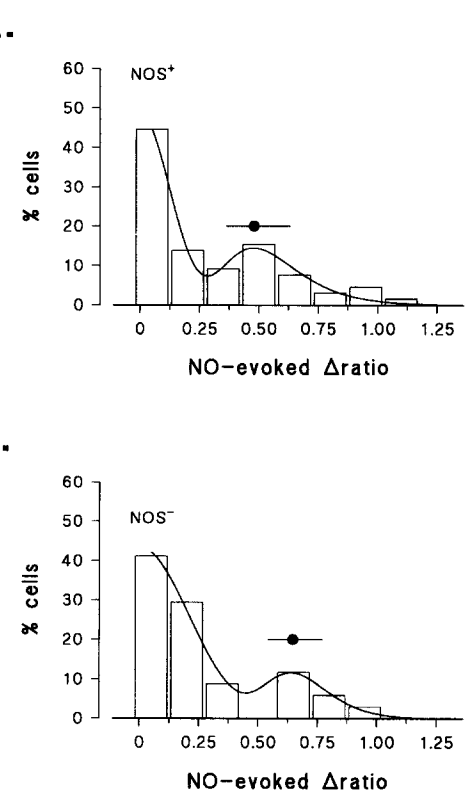

Fig. 5. Distribution of NO-donor-responsive cells between NOS^+ and NOS^- chromaffin cells. (A, B) Frequency histograms showing the distribution of 100 μM molsidomine evoked increases in Δ ratios in NOS^+ and NOS^- chromaffin cells. The average ratio increase was significantly higher in NOS^- chromaffin cells ($P<0.01$; Student's *t*-test).

to whether NO responses are correlated to the adrenergic/noradrenergic or NOS^+/NOS^- phenotype of chromaffin cells. Immunoidentification of NO-responsive cells revealed that they were relatively more frequent among adrenergic cells than among noradrenergic cells. In the same way, the expression of NOS was not an unambiguous marker for the identification of NO-responsive cells, although $[Ca^{2+}]_i$ responses to NO donors were more frequent among NOS^- cells. As a result of the cumulative bias of the distribution of responses to NO donors in adrenergic/noradrenergic and NOS^+/NOS^- cells, NO-evoked $[Ca^{2+}]_i$ transients were significantly less abundant among NOS^+ noradrenergic cells. Thus, the proportion of adrenergic/noradrenergic cells (as well as NOS^+/NOS^- cells) in a mixed population could significantly affect the average signal elicited by NO donors.

In contrast to the incomplete segregation of responses to NO donors, we show here a clearly asymmetric distribution of NOS immunoreactivity among adrenergic and noradrenergic cells. NOS immunoreactivity is mainly (82 ± 2%) localized in $PNMT^-$ chromaffin cells, despite noradrenergic cells being in lower number than adrenergic cells in the bovine adrenal gland. This result agrees with and extends previous data indicating that NOS was present only in a subpopulation of chromaffin cells in human[15] and, not so clearly, in rat adrenal medulla.[9] These results point out that the information flow carried by NO within adrenal medulla travels mainly from noradrenergic cells (most NOS^+ cells are

Table 3. Distribution of nitric oxide-donor responsive cells among nitric oxide synthase-positive/negative and adrenergic/noradrenergic chromaffin cells

	Adrenergic	Noradrenergic	
NOS^+	73 ± 6% (45/62)	35 ± 11% (7/20)	$P<0.05$
NOS^-	59 ± 8% (20/34)	67 ± 16% (6/9)	N.S.

Data collected from nine different stimulations in three independent experiments. Statistical analysis: S.D. values were obtained assuming a binomial distribution. The whole table was tested for equal proportion hypothesis by both ANOVA and a Pearson χ^2-test. Both tests gave significant results at $P=0.05$, indicating unequal distribution of NO-donor responsive cells. *P* values quoted correspond to a within-rows χ^2-test. Results in parenthesis represent the number of responding cells against the total cell number ($n=125$ cells).

PNMT$^-$) to adrenergic cells (they are more abundant and mostly NOS$^-$ and responsive to NO donors). In other words, NO may have a paracrine role, with noradrenergic cells specialized in the synthesis of NO whereas adrenergic cells constitute mainly the target of NO action. This view is stressed considering the short life and range of action of NO in a well-irrigated tissue like adrenal medulla.

CONCLUSION

Our results emphasize the importance of studying even relatively simple systems such as adrenomedullary cultures at the cellular level and highlights the functional importance of chromaffin cell heterogeneity in relation to NO actions.

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