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Journal of Insect Physiology 53 (2007) 1188–1197
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

The role of *N*-β-alanyldopamine synthase in the innate immune response of two insects

Julieta Schachter[1], Martín M. Pérez[1], Luis A. Quesada-Allué*

Department of Biological Chemistry, FCEyN, University of Buenos Aires, IIBBA-CONICET and Fundación Instituto Leloir, Patricias Argentinas 435, Buenos Aires 1405, Argentina

Received 30 March 2007; received in revised form 13 June 2007; accepted 14 June 2007

Abstract

Insects trigger a multifaceted innate immune response to fight microbial infections. We show that in the yellow mealworm, *Tenebrio molitor*, septic injuries induce the synthesis of *N*-β-alanyldopamine (NBAD), which is known as the main sclerotization precursor of insect brown cuticles. We demonstrate that NBAD synthase is induced in the epidermis of the mealworm and of the Medfly, *Ceratitis capitata*, by infection with *Escherichia coli*. Our results indicate that synthesis of NBAD seems to be a novel component of the overall innate immune response in insects.

Keywords: *N*-β-alanyldopamine; Insect immunity; β-alanine; *Ceratitis capitata*; *Tenebrio molitor*

1. Introduction

In addition to the physical cuticle barrier, insects display three main strategies of defense to avoid infection (Hoffmann, 2003; Royet et al., 2005). The first line of response to microbial entry is mediated by the rapid action of phenoloxidases, which are present in the integument and in the hemolymph as inactive pro-enzymes that are converted to active form by a protease cascade (Ashida and Brey, 1995). These generate quinones that lead to localized melanization and cross-linking, eventually forming melanotic structures that encapsulate invaders (Ashida and Brey, 1995; Nappi and Ottaviani, 2000). Then, a cellular defense mechanism is triggered: within minutes of infection, hemocytes start to display a phagocytic behavior, interacting with foreign pathogens or materials and clearing invaders from the hemocoel (Agaisse et al., 2003; Ferrandon et al., 2004; Hoffmann, 2003; Royet et al., 2005). The third line of defense is humoral and requires several hours for full expression, involving the induced synthesis of antimicrobial peptides and proteins by both epidermis and fat body (Hoffmann and Reichardt, 2002; Tzou et al., 2002).

Hopkins et al. (1998) reported that catecholamine biosynthesis seems transiently stimulated in the case of parasitized tobacco hornworm (*Manduca sexta*) larvae. These authors showed that, unexpectedly, *N*-β-alanyldopamine (NBAD), which is the main sclerotization precursor of insect brown cuticle, was induced by the parasitoid. Moreover, Kim et al. (2000) observed an increase of NBAD in the yellow mealworm beetle *Tenebrio molitor* infected with bacteria. The synthesis of this β-alanyl (β-ala) derivative is periodically induced in insect epidermis, but only at the time of molting (Hopkins and Kramer, 1992; Kramer and Hopkins, 1987; Pérez et al., 2002). In contrast, we recently reported that NBAD also is present in neural tissue, in a constitutive manner (Pérez et al., 2004). Here, we demonstrate in *T. molitor*, and in the Medfly, *Ceratitis capitata*, a novel immunological response that, unexpectedly, triggers the induction of the epidermal enzyme NBAD-synthase (NBAD-S) during intermolt.

Abbreviations: β-ala, β-alanine; NBAD, *N*-β-alanyldopamine; NBAD-S, *N*-β-alanyldopamine synthase

*Corresponding author. Tel.: +54 11 5238 7500; fax: +54 11 5238 7501.
E-mail address: lualque@iib.uba.ar (L.A. Quesada-Allué).

[1]J.S. and M.M.P. contributed equally to this work.

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doi:10.1016/j.jinsphys.2007.06.010

2. Materials and methods

2.1. Insects

T. molitor were reared in stale bread with oatmeal and a slice of potato as a source of water. Wild type *C. capitata* (“INTA Arg 17”) strain and the mutant strain *niger* (*nig*[1]) were reared in a growth chamber at 23 °C and 75% humidity with an artificial day of L16:D8 h. We designed a novel squash-based fly food that contained: 1 portion (w/w) crude and 1 portion boiled organic squash, 0.2 portions live yeast powder, 1 portion pre-cooked corn meal, 0.5 portion sucrose, 1. 7 g/L sodium benzoate, 0.5 g/L Nipagin M and 15% (v/v) standardized apricot juice (Baggio). The mixture was adjusted to pH 5.5 with 10% HCl.

2.2. Chemicals

All the available biogenic amines and/or standards were from Sigma Chem. Co. [1-^{14}C]β-alanine (55.0 μCi/μmol) was from New England Nuclear. *N*-β-alanyldopamine and other standards were generously synthesized by Dr. J.M. Aguirre (Luján University, Argentina) as described in Yamasaki et al. (1990) with slight modifications. Sarcophagine (*N*-β-alanyltyrosine) was generously synthesized by Dr. J. Drijfhout from the Medical Center of Leyden University, the Netherlands.

2.3. In vivo NBAD biosynthesis

T. molitor larvae were injected through the abdominal arthrodial membranes with 10 μL insect Ringer’s solution containing [1-^{14}C]β-ala (30,000 cpm, 250 pmol) with or without *Escherichia coli* DH 5α (~10^7 cells/mL). When leaks or damage occurred, the insect was discarded. In all experiments, controls included punctured insects (mock injection) and those injected with sterile insect Ringer’s solution alone. Each series of experiments was carried out at least in triplicate. The injected animals were maintained at 23 °C for 20 h, chilled on ice and then dissected. The hemolymph was separated and the internal organs, heads and integumental carcasses were separately ground under liquid nitrogen in small mortars and homogenized in glass–glass or Teflon–glass micro-homogenizers. Percloric acid was added to hemolymph and to the tissues homogenates to a final 2% concentration and the precipitated proteins were pelleted at 16,000*g*. Soluble extracts were processed as described below. The identity of the synthesized [^{14}C]NBAD in these and in the *in vitro* experiments was assessed by co-chromatography with authentic NBAD in HPLC (Pérez et al., 2002, 2004; Wappner et al., 1996a, b) (Econosphere-C-18 columns, Altech; 2 different buffers) and TLC (several solvents) and by hydrolysis in 1.2 N HCl during 2 h at 98 °C and further identification of the liberated [^{14}C]β-ala. Silica Gel TLC chromatograms shown in Figs. 1 and 3 were developed in methyl-ethyl-ketone:propionic acid:water (40:13:11) and the labeled material was located by autoradiography (X-OMat-Kodak).

2.4. Synthase assay and product characterization

Cell-free synthase activities were measured as previously described (Pérez et al., 2002, 2004). The reaction mixture contained 5 mM $MgCl_2$, 2 mM ATP, 0.1 mM dopamine and 30,000 cpm of [1-^{14}C]β-ala (55.0 μCi/μmol) (250 pmol) in 50 mM sodium borate buffer, pH 8.3. The reaction was started by addition of 10 μg enzymatic protein in a final volume of 50 μL and stopped by addition of perchloric acid to 2% final concentration. The samples were centrifuged at 16,000*g* and the supernatant immediately analyzed. Catecholamine derivatives were isolated using 150 mg of chromatographic acid alumina (Sigma) resuspended in 200 μL of 1 M Tris–HCl buffer, pH 8.7. The alumina slurry was added to the experimental tubes and the mixture was incubated for 15 min at ambient temperature with continuous shaking. The suspension was poured into small polyethylene columns and the percolate recovered in a scintillation vial. The alumina was washed several times with 1.5 mL of 0.1 M Tris–HCl, pH 8.7, and the first eluates were recovered in scintillation vials. Finally, the catecholamines were eluted by addition of 1.7 mL of 1 M acetic acid and recovered in a scintillation vial. For qualitative analysis, the samples were lyophilized and both the eluate and the material retained by the alumina were analyzed by reverse-phase HPLC as previously described (see above). Radioactivity in the samples and in the HPLC fractions of both the catecholamine derivatives bound to alumina and of the percolates containing the remaining [1-^{14}C]β-ala and eventually derivatives of biogenic amines were counted in a Rackbeta 1214 Pharmacia liquid scintillation counter using Ultima-gold (Packard) as scintillant. Protein concentration was determined using the Bradford reagent (Sigma) and BSA as standard. Aliquots of the synthesized bound radiolabeled material behaving as NBAD in HPLC were analyzed in mono- and bi-dimensional Silica Gel TLC Chromatography with different solvents together with standards.

2.5. NBAD-S induction

NBAD-S was induced by injection of microorganisms or polysaccharides in insect Ringer′s. In a typical experiment, *T. molitor* larvae received 10–13 μL of an $OD_{595} = 0.5$ suspension containing bacteria (~100–130,000 bacteria/insect); whereas *C. capitata* adults were injected with 1 μL of a 10-fold dilution of a similar suspension (~1000 bacteria/insect). Carcasses and other tissues were dissected and processed as above. Crude homogenates were used as a source of enzymes but, in conjunction with previous reports (Pérez et al., 2002, 2004), after three purification steps, the ecdysone-inducible and the bacterial-induced NBAD-S activities of both *T. molitor* and C. *capitata* were completely lost. Therefore, slightly purified enzymatic

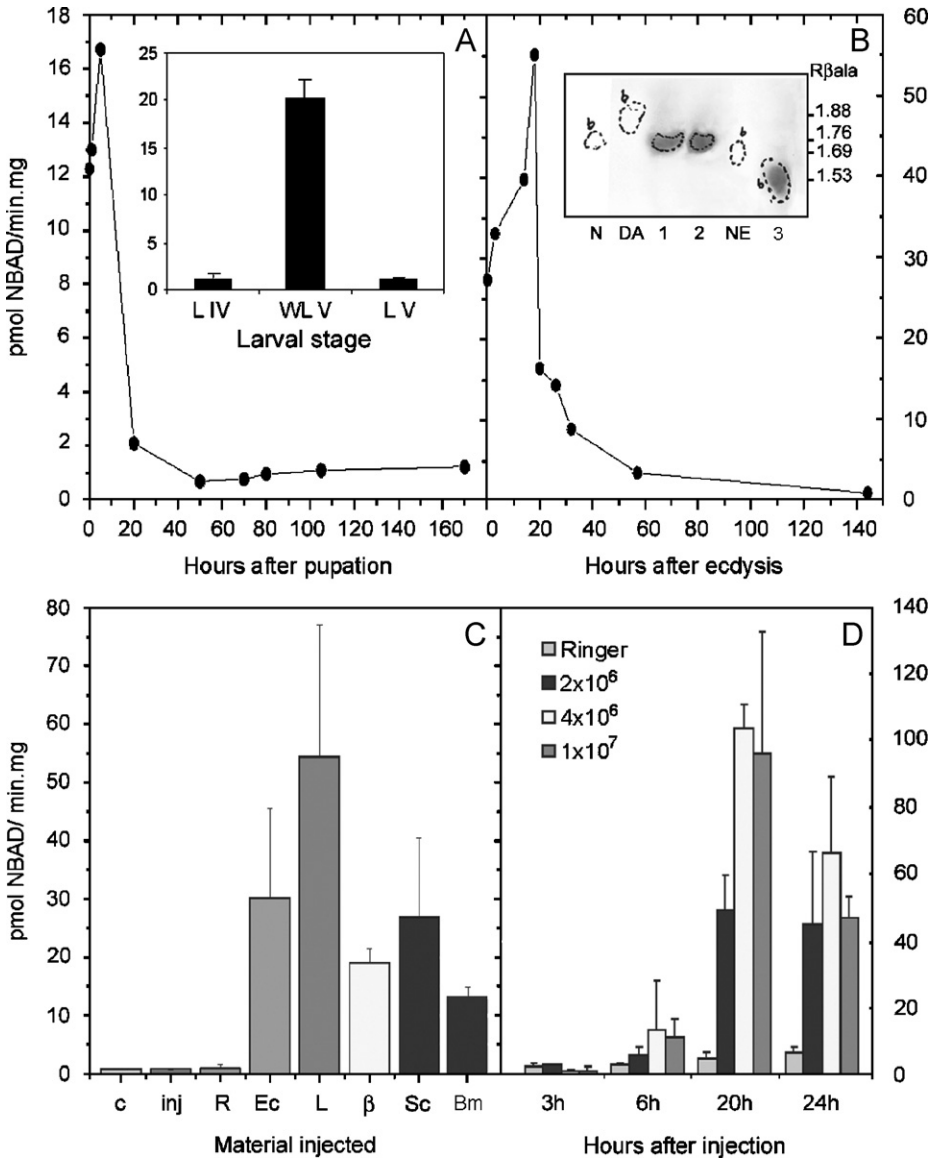

Fig. 1. Enzymatic activity of NBAD-S in *Tenebrio molitor*. Substrates were [1-^{14}C]β-alanine and dopamine. The product NBAD was analyzed as described in Section 2. Panel (A): activity of NBAD-S in pupae; time zero indicates the onset of pupation. *Inset*: enzymatic activity of non-sclerotizing larva IV (LIV), sclerotizing white larva V (WLV) and non-sclerotizing larva V (LV). Panel (B): activity after adult ecdysis; time zero indicates ecdysis. *Inset*: TLC/autoradiography of the labeled material; standards are: N: NBAD; DA: dopamine; NE: norepinephrine; 3: [^{14}C]β-alanylnorepinephrine; 1,2: labeled products; Rβ_{ala}: relative mobility to β-alanine. Panels (C,D): induction of NBAD-S after microbial challenge. The insects were injected with 10 μL of Ringer containing live cells (10^7 *E. coli* cells/mL) or other antigens and analyzed for NBAD-synthase induction. (C) Enzymatic activity 20 h after injection with: EC: *E. coli*; L: bacterial lipopolysaccharide; β: algal β-1-3 glycan; Bm: *Bacillus megaterium*; Sc: the insects were pricked with a needle soaked with *S. cerevisiae* cells; c: control; inj: injured with a dry needle; R: sterile Ringer control. (D) Time-dependent induction of NBAD-S with different levels of *E. coli* infection (10 μL of the indicated cells/mL suspensions).

extracts were prepared exactly as described in Pérez et al. (2002). Cell-free synthase activities were measured as above. The radiolabeled substances bound to alumina were analyzed in HPLC, diluted with a scintillation fluid (Packard Ultima Gold) and counted in a Pharmacia 1214 Rackbeta scintillator.

2.6. Antimicrobial activity of NBAD

Bacteria *E. coli* DH 5α and *Bacillus megaterium* were grown overnight in liquid Luria Bertani medium (LB medium) and yeast *Saccharomyces cerevisiae* PRY225 in YCD-WV medium, up to exponential growth phase. To determine the antimicrobial property of NBAD, several series of assays were performed in sterile 96-well microplates. The amount of available NBAD (non-commercial) is a bottleneck for this kind of experiments. An inoculum from a mid-logarithmic phase culture was transferred to fresh medium in wells starting at $A_{595\,nm} = 0.002$, in the presence of different concentrations of NBAD. Microbial growth in the permanent presence of NBAD or sarcophagine was assessed by an increase in $A_{595\,nm}$, while

incubating the bacterial culture for 24 h at 37 °C and the yeast culture at 28 °C. Oxidation of NBAD was estimated by color generation in blank tubes at $A_{595\,nm}$ and the value of net bacterial growth obtained by substraction. Inhibition of growth was estimated (Fig. 4, inset) by plating 20 μL aliquots of microbial cultures on 10 cm Petri dishes with nutrient agar. The number of colony forming units (CFU) was determined after overnight culture at 37 °C. Control experiments were run in the absence of NBAD with or without the presence of neutral dipeptides.

3. Results

3.1. Beetle NBAD-S characteristics

To measure, for the first time in coleopterans, the enzymatic activity responsible for the synthesis of NBAD in epidermis, we prepared extracts from different life-cycle stages of the yellow mealworm, *T. molitor*. Fig. 1A,B shows that, as expected, the beetle enzyme was periodically induced at the time of molting. The inset in Fig. 1A shows the rate of synthesis during the *T. molitor* larval IV–V intermolt (LIV), which was 1.13 ± 0.52 pmol NBAD/mg min whereas immediately after molt ("white" larva V, WLV), the rate was 20.32 ± 1.85 pmol NBAD/mg min, and then dropped (larva V, LV). During the transition from larva V to pupa (Fig. 1A) and from pupa to adult (Fig. 1B), the enzymatic activity again was transiently expressed and then returned to trace level. The respective highest rates of synthesis were 16.7 ± 0.7 pmol NBAD/mg min at 5 h after puparium formation (Fig. 1A) and 55.0 ± 3.2 pmol NBAD/mg min at 15 h after imago ecdysis (Fig. 1B). The identification of the labeled material as [^{14}C]NBAD was made by co-chromatography with standards in HPLC (same HPLC profile as in Fig. 2A), and in TLC (inset in Fig. 1B). The identity was indirectly confirmed by recovery of [^{14}C]β-ala when the material from HPLC was hydrolyzed (same result as in the inset in Fig. 2A). The overall characteristics of the enzyme were similar to those of the NBAD-S from *C. capitata* (Pérez et al., 2002) and identical to those of the *T. molitor* bacteria-induced enzyme (see below). Thus, as expected, the synthesis of the sclerotization precursor NBAD in coleopteran epidermis is periodic and exclusively

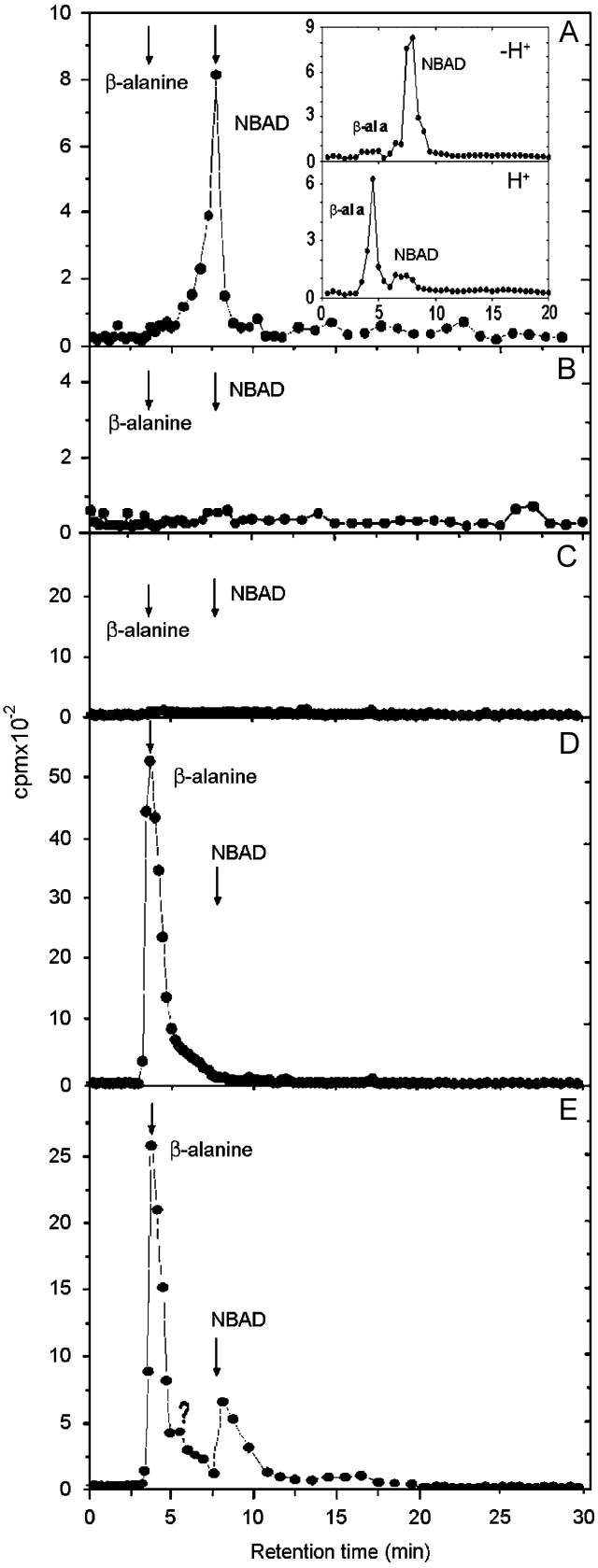

Fig. 2. *In vivo* induction of NBAD synthesis. Non-sclerotizing *T. molitor* larvae V were injected with a micro-needle. Injections of 13 μL insect Ringer contained [1-^{14}C]β-alanine as substrate tracer with or without bacteria. After 20 h, the soluble substances from carcasses homogenates were extracted and analyzed in HPLC together with standards. (A) Injection containing a bacterial suspension (10^7 *E. coli* cells/mL). Alumina-bound material was counted and analyzed. *Inset*: boiling (2 h, 98 °C) of the [1-^{14}C]β-alanine-labeled material with (bottom) or without (top) 1.2 N HCl. (B) Control: same as in (A) without bacteria. (C,D) Injection was as in (B) but with 1 mM dopamine. Analysis of alumina bound (C) and unbound (D) material. (E) Injection as in (A) but with additional 1 mM tyramine. (?) indicates traces of unknown substances.

associated to molt, as previously demonstrated in dipterans (Pérez et al., 2002; Wappner et al., 1996a, b).

3.2. In vivo induction of NBAD after microbial challenge

Non-sclerotizing intermolt larvae V of *T. molitor* were injected with 10 μL of sterile insect Ringer's solution containing live Gram negative *Escherichia coli* (10^7 cells/mL) and [1-^{14}C]β-ala (30,000 cpm). The insects were maintained for 20 h at 23 °C. After removing the head and internal tissues, the carcasses (cuticle plus epidermis and longitudinal muscles fibers) were reduced to powder under liquid N_2 and homogenized. After centrifugation, the soluble material was poured into an acid alumina column equilibrated with pH 8.7 buffer. The radioactivity in the material bound to alumina, therefore behaving as a catecholamine (Eriksson and Persson, 1982), was eluted, quantified and analyzed together with standards using HPLC and TLC (Section 2 and Pérez et al., 2002). The infected insects contained a [^{14}C]-labeled substance that behaved as NBAD (69.7 pmol/mg protein) as judged from co-chromatography with standards in HPLC (Fig. 2A) and TLC (not shown). After acid hydrolysis in 1.2 N HCl for 2 h at 98 °C, which is the condition for acid hydrolysis of NBAD, most of the recovered radioactivity was undistinguishable from β-ala while the original peak ([^{14}C]NBAD) diminished (inset in Fig. 2A). No NBAD was synthesized after identical injections without bacteria (Fig. 2B). No significant [^{14}C]-labeled catecholamine-like material was detected in internal organs (see below). As expected (Pérez et al., 2004), NBAD was constitutively synthesized in the head both without and with bacterial challenge.

Since the bacterial induction of dopa decarboxylase has been reported (Kim et al., 2000), the possibility exists that an excess of dopamine production might lead to conjugation with [^{14}C]β-ala even if only minimum levels of NBAD-S were present. However, after injection of sterile insect's Ringer containing 1 mM dopamine in addition to [^{14}C]β-ala, no NBAD was synthesized (Fig. 2C). The analysis of the material not retained by the alumina column, demonstrated that after 20 h, most of the recovered soluble radioactive material was the injected [^{14}C]β-ala (Fig. 2D), thus confirming that the induction of NBAD (Fig. 2A) was due to bacterial challenge triggering an increase in NBAD-S activity and not to dopamine excess.

To gain insight into the regulation of β-ala derivatives synthesis after bacterial challenge, *in vivo* experiments were performed where 1 mM tyramine was injected to compete with the endogenous dopamine (Fig. 2E). No *N*-β-alanyl derivative of tyramine was detected and most of the synthesized material was identified as NBAD although small amounts of substances behaving like *N*-β-alanyl-norepinephrine and other unknown derivatives were detected (Fig. 2E). The scarce amount of these minor radioactive products avoided further characterization.

3.3. Induction of NBAD-S due to bacterial challenge

Fig. 1C shows the induction of NBAD-S activity in extracts from non-sclerotizing larvae V of *T. molitor*. Twenty hours after injection of 10 μL of insect Ringer's containing live *E. coli*, the enzymatic activity was measured in the internal organs, hemolymph and in the beheaded integumental carcass (mostly cuticle plus epidermis plus remaining muscle fibers). The epidermis enzyme from carcasses was highly induced in response to the infection (Fig. 1C), showing an activity of 69.7 pmol NBAD/mg min whereas no induction of NBAD-S was detected in hemolymph (3.3 pmol/mg min), hemocytes (0.0 pmol/mg min) and in the rest of internal organs (5.1 pmol/mg min). Bacterial LPS (0.1 μg/insect) and an algal β(1→3)glycan (0.1 μg/insect) were also elicitors of the epidermal NBAD-S (Fig. 1C); which was also induced when *T. molitor* was pricked with a needle soaked with yeast (*S. cerevisiae*) cells (Fig. 1C). Preliminary results on infection with Gram-positive *B. megaterium* also showed the expression of NBAD-S (Fig. 1C). A small increase in NBAD-S activity was detected as early as 6 h after injection of *E. coli* whereas the maximum enzyme activity was reached 20 h after injection (Fig. 1D). With low-bacterial challenge the same amount of active enzyme was present at 20 and 24 h whereas under intermediate and higher infections the amount of active NBAD-S was higher at 20 h than at 24 h (Fig. 1D). No induction occurred when the insects were injured or injected with aseptic Ringer's solution (Fig. 1C).

Taking advantage of the well-established timing of life-cycle events in the Medfly, *C. capitata* (Rabossi et al., 1992), we micro-injected bacteria (1 μL of 1.6×10^6 *E. coli* cells/mL) in non-sclerotizing (1 week after ecdysis) adults of this fly. Only traces of NBAD-S activity can be detected in the integument of controls at this point of the life cycle (0.45 pmol NBAD/min mg; Fig. 3A) but after injection of live *E. coli*, the activity of the Medfly NBAD-S in extracts from the abdominal and thoracic epidermis was 13 pmol/min mg, whereas the activity of the constitutively expressed brain enzyme remained constant (Fig. 3A). The melanic mutant *C. capitata niger*^{1} is unable to express NBAD-S either in epidermis at molt or in brain (Pérez et al., 2002, 2004). As expected, no NBAD-S was induced in this mutant after *E. coli* injection (Fig. 3B) and the mortality both 20 and 24 h after the challenge was always significantly higher than that of wt (Fig. 3C).

3.4. Characteristics of the microbe-induced epidermal enzyme

The characteristics of the bacteria-induced *T. molitor* enzyme were found to be identical to those of the canonical (20-OH-ecdysone-induced) epidermis enzyme shown in Fig. 1A,B. NBAD-S required divalent cations (Fig. 4A), Mg^{2+} being the best (Fig. 4A,B). As described for *C. capitata* epidermis and brain enzymes, cobalt and

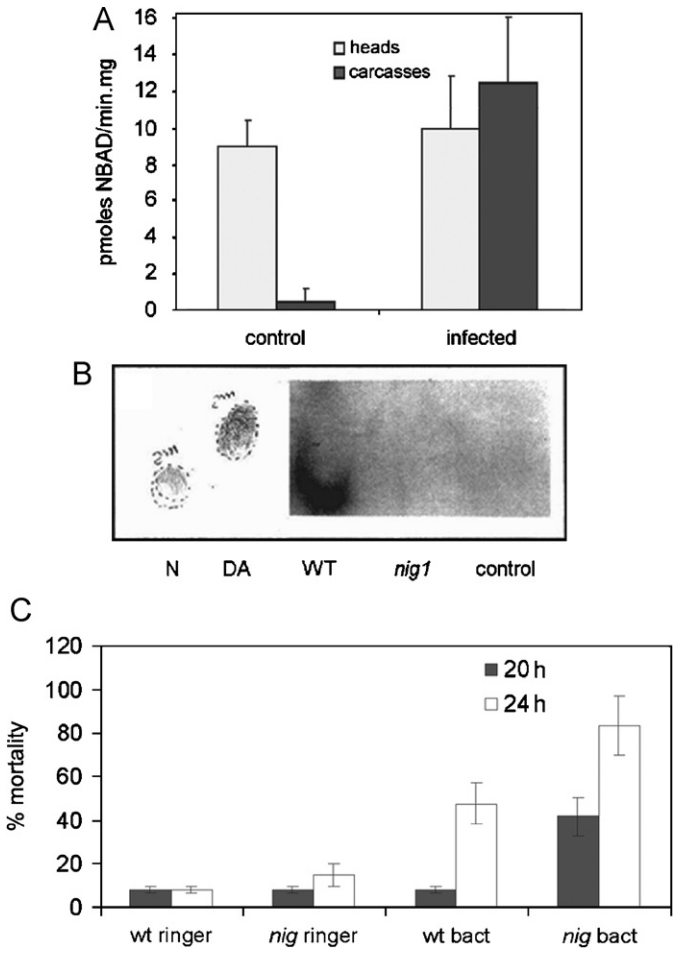

Fig. 3. Induction of NBAD-S in *C. capitata* adults. (A) NBAD-S activity in heads and body carcasses of adult WT flies, 20 h after *E. coli* injection (1 μL of 1.6×10^6 cells/mL). (B) TLC autoradiography of the conjugates synthesized by homogenates from body carcasses of WT and *niger*^{1} after bacterial challenge. Controls do not receive bacteria. Standards: N and DA as in Fig. 1. (C) Increased mortality of the *niger*^{1} (*nig*) mutant.

calcium were not suitable co-factors for the *T. molitor* enzyme whereas 5 mM of Zn^{2+}, Ni^{2+} or Cd^{2+} were inhibitors even in the presence of 10 mM $MgCl_2$ (Fig. 4A). ATP was an absolute requirement (Fig. 4B), with an optimal concentration of 2 mM. The optimum pH was 8.4 (Fig. 4C) and the kinetics appeared Michaelian with a V_{max} of 330 pmol/min mg and an apparent K_m for β-ala of 130 μM (Fig. 4D). Similar to what was shown previously in the molt-related epidermis enzyme of dipterans (Pérez et al., 2002), the coleopteran NBAD-S has a broad substrate specificity. Cell-free experiments demonstrated that the bacteria-induced *T. molitor* enzyme was able to synthesize conjugates of norepinephrine (*N*-β-alanylnorepinephrine, NBANE), (Fig. 5A), as well as of tyramine (*N*-β-alanyltyramine, NBATA), (Fig. 5B) and of octopamine (*N*-β-alanyloctopamine, NBAOA), (Fig. 5C).

All the above characteristics are similar to those previously demonstrated for the ecdysone-induced *C. capitata* epidermis enzyme (Pérez et al., 2002), and for the constitutive *C. capitata* brain enzyme (Pérez et al., 2004). They were identical to those of the above-described *T. molitor* molt-dependent epidermis enzyme (see Section 4).

3.5. Antimicrobial properties of NBAD

The estimated oxidation half-life of NBAD in bacteria-free control cultures was 4 h, as judged by the increase in absorbance at 595 nm. Liquid cultures of *E. coli* showed that 3 mM NBAD completely abolished the growth during 24 h, even if at this time, almost all the NBAD was presumably oxidized (Fig. 6A,B). NBAD was more efficient growth inhibitor than sarcophagine (Fig. 6A), previously reported as bactericidal (Kano and Natori, 1984; Meylaers et al., 2003). The inset in Fig. 6A shows the CFU of *E. coli* when 20 μL aliquots of liquid cultures were plated on agar after 3, 6 or 9 h with NBAD. Liquid cultures containing 3 mM NBAD do not generate colonies after overnight culture, whereas in 2 mM cultures, after 9 h, viable bacteria were present (inset in Fig. 6A). Preliminary results showed that 3 mM NBAD also inhibited the growth of the Gram-positive *B. megaterium* (Fig. 6C) and slightly retarded that of the yeast *S. cerevisiae* (Fig. 6D). When 20 mM carcinin, an antioxidant, was included in the culture media together with 2–5 mM NBAD, the bacteriostatic effect of the latter was partially reversed (not shown), thus strongly suggesting that quinones are the active molecules.

4. Discussion

Until recently, it was accepted that NBAD-S is periodically induced in epidermis by the molt hormone, 20-OH-ecdysone, only at the time of molting, and is exclusively involved in sclerotization and pigmentation of cuticle (Hopkins and Kramer, 1992). In this report, we have demonstrated for the first time the bacterial induced expression of the otherwise repressed integumental NBAD-S; both in intermolt larvae of *T. molitor* (Fig. 1C,D) and non-sclerotizing adults of *C. capitata* (Fig. 3). The small activity detected in internal organs (see Section 3) may be attributed to thoracic and abdominal ganglia, which, as previously demonstrated (Pérez et al., 2004), constitutively express neuronal NBAD-S. The requirements and characteristics of the bacterial-induced epidermal NBAD-S from *T. molitor* (Fig. 4) were identical to those of the canonical enzyme induced at the time of molting and similar to those of the *C. capitata* enzymes studied in our lab (Pérez et al., 2002, 2004). Since during intermolt in the absence of bacterial challenge, no *in vitro* NBAD-S activity and no *in vivo* NBAD were detected in the epidermis, we can assume that the experimental infection probably triggers the expression of the respective genes coding for NBAD-S. Alternatively, the activation of a zymogenic form of the enzyme, already present in the epidermis, might be postulated, but no evidence whatsoever of this possibility has been found. The lack of NBAD-S induction in the point-mutated *niger*^{1} indirectly confirms

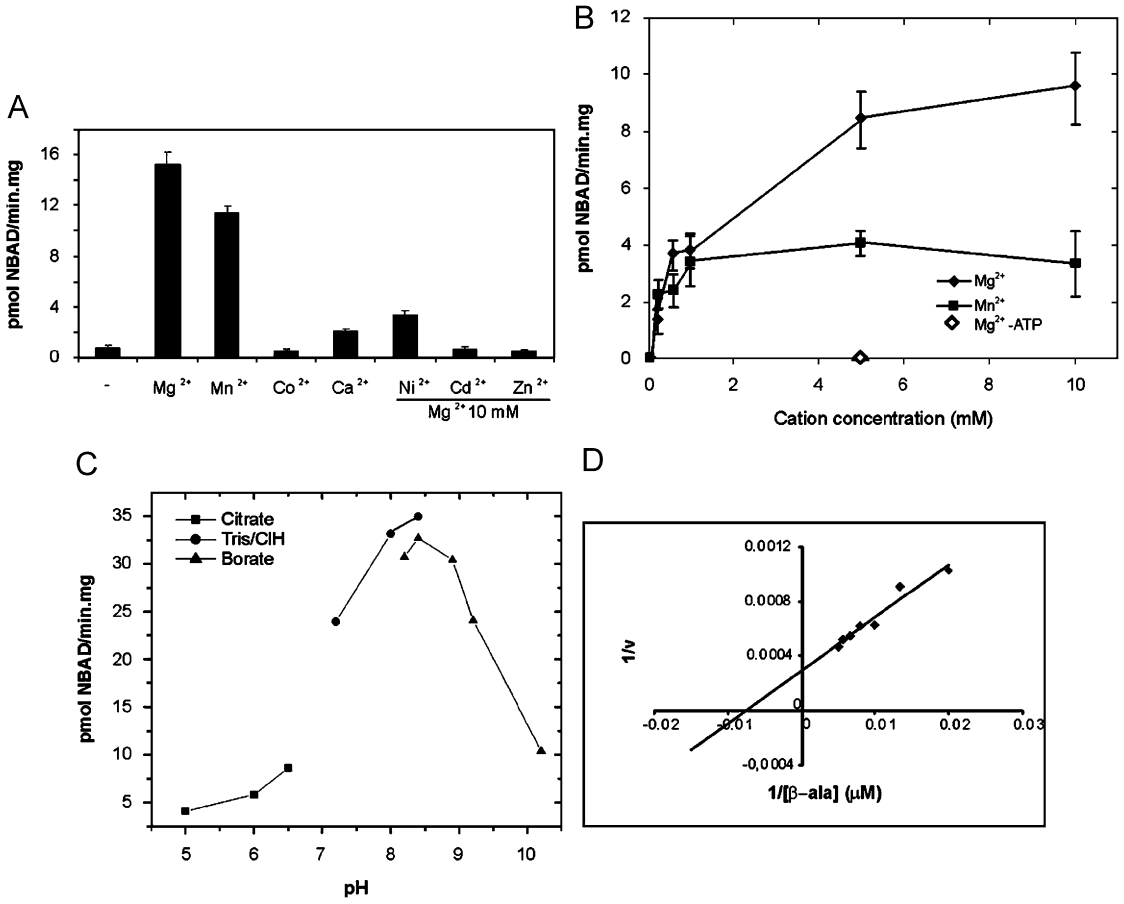

Fig. 4. *In vitro* activity of the bacteria induced *T. molitor* NBAD-S: (A) stimulation by 5 mM divalent cations, (B) Mg^{2+} and Mn^{2+} concentration-dependent activity, (C) optimum pH, (D) Lineweaver–Burk plot for β-ala substrate.

that the two *C. capitata* epidermal NBAD-S activities respectively induced in WT by 20-OH-Ecdysone at molt (Pérez et al., 2002; Rabossi et al., 2000; Willis, 1974) and by *E. coli* challenge (this paper) as well as the brain enzyme (Pérez et al., 2004) are coded by the same single gene. The biochemical data strongly suggest that this is also true in the case of *T. molitor*.

Our results explain published data showing that the injection of Gram-negative bacteria in the hemocoel of *T. molitor* (Kim et al., 2000) and parasitism by wasps of *M. sexta* larvae (Hopkins et al., 1998) appear to increase the amount of circulating NBAD. We know from previous studies (Pérez et al., 2002) that sarcophagine is another alternative product of NBAD-S *in vitro* activity. The analysis of the radioactive material from the *in vivo* experiments that was not retained by alumina columns showed that apparently no sarcophagine or other derivatives were synthesized in response to bacterial challenge.

In holometabolous insects, no dopamine was usually detected in hemolymph during intermolt (Hopkins and Kramer, 1992). Our results showed that in the absence of bacterial challenge, when 1 mM exogenous dopamine was injected, thus putative activation of dopa-decarboxylase was mimicked (Kim et al., 2000), no NBAD was synthesized (Fig. 2C). This indicates that no active NBAD-S was available and that an eventual dopa-decarboxylase induction would not be responsible for microbial induction of NBAD synthesis. Injection of tyramine to compete with endogenous dopamine revealed (Fig. 2E) that in spite of the wide substrate specificity of the bacteria induced NBAD-S when tested *in vitro* (Fig. 5; see Pérez et al., 2002, 2004) the main product generated *in vivo* was NBAD.

The induction triggered by Gram-negative *E. coli* was clearly demonstrated but we also obtained preliminary positive results with different microbes and polysaccharides (Fig. 1C). Therefore, further experiments should establish the specificity of the induction, if any.

The induction timing of NBAD-S, which can be detected only after 6 h of microbial challenge and can reach maximum activity after 20 h (Fig. 1D), is somehow similar to the reported timing of antibacterial peptides synthesis (Lemaitre et al., 1997). However, in that systemic response, fat body cells synthesize the antimicrobial peptides precursors whereas in our studies no catecholamine-like labeled derivatives were found associated with the fat body; and only the epidermis-rich carcass showed an active NBAD-S. The timing similarity of both responses seems to

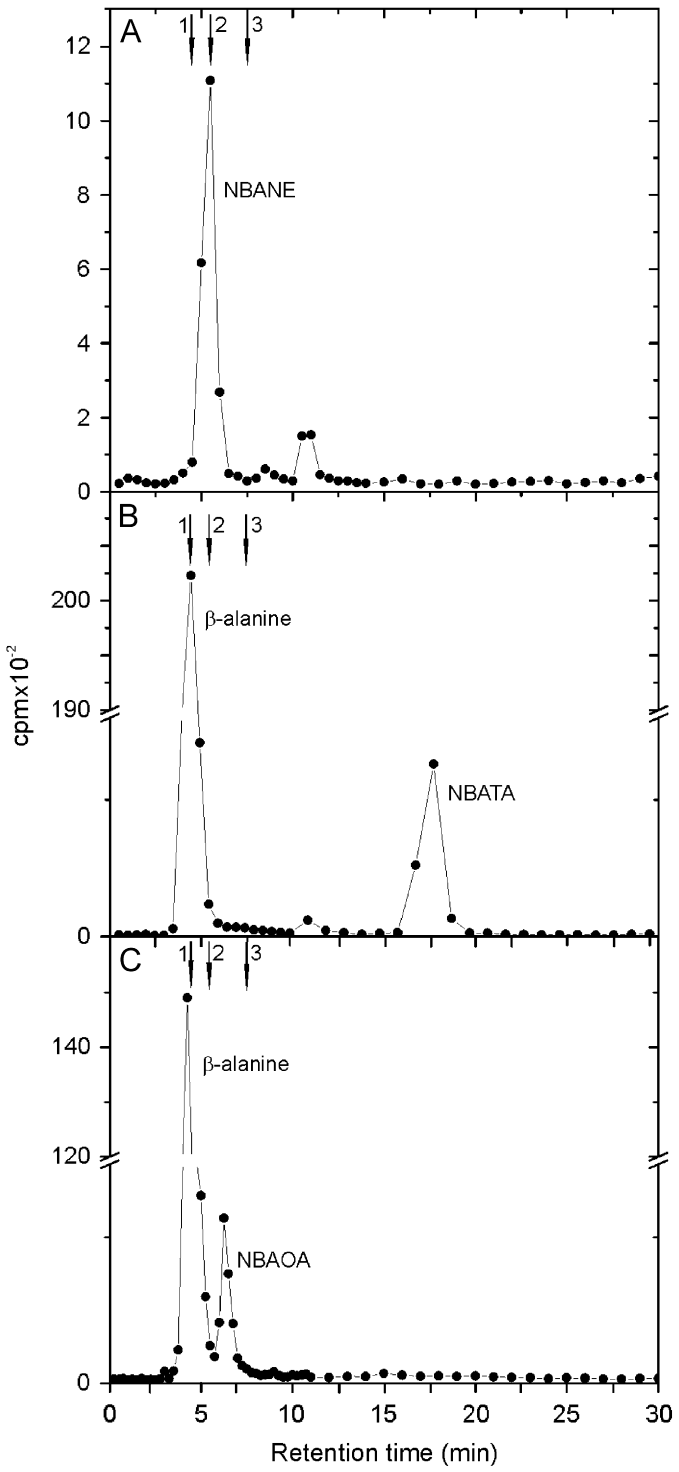

Fig. 5. HPLC of the products obtained after incubation of the induced *T. molitor* NBAD-S with nor-epinephrine (A), tyramine (B) or octopamine (C) instead of dopamine. Arrows—1: β-ala; 2: NBANE and 3: NBAD.

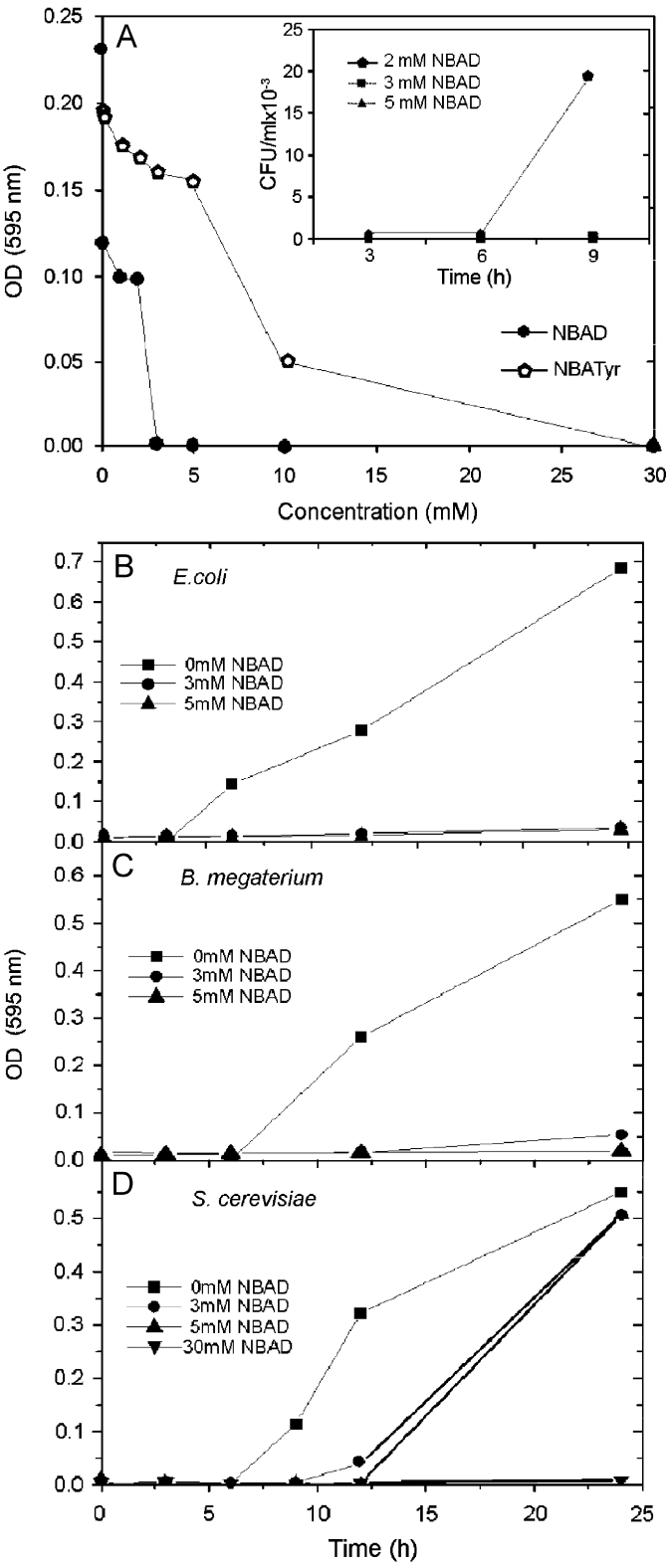

Fig. 6. Antimicrobial activity of NBAD: (A) concentration-dependent inhibition of *E. coli* cultures in the presence of NBAD (●) or sarcophagine (NBATyr, ⬠). *Inset*: time-dependent colony formation capacity after exposition to NBAD during the indicated times. (B–D) Inhibition by NBAD of *E. coli* growth (B), *B. megaterium* growth (C) and *S. cerevisiae* growth (C).

reinforce the idea that a link between hemocyte-mediated phagocytosis and immune responses may exist (Brennan et al., 2007).

It is assumed that synthesis of antimicrobial peptides by epidermis is always local, restricted to the cells surrounding a wound (Hoffmann, 2003). In the NBAD-S induction

reported here, since the bacteria are injected into the hemocel, we assume that we are looking at a generalized activity taking place in all the epidermis cells through a typical systemic response. The function of NBAD in the immune response is not known, although it can be hypothesized that the action of phenol-oxidase enzymes could give rise to reactive quinones. The physiological levels of NBAD in hemolymph during sclerotization of the *M. sexta* cuticle were 4.16 mM, as measured by Hopkins et al. (1982). During sclerotization of *C. capitata* puparium, we detected 0.3–1.0 mM levels of NBAD (Wappner et al., 1996a, b). The preliminary results in this paper (Fig. 6) show that millimolar levels of NBAD can inhibit microbial growth. The minimum concentration levels of NBAD required for *in vitro* bacterial growth inhibition are similar or lower than those reported for other insect low-molecular weight antibacterial molecules like sarcophagine (Fig. 6A; Meylaers et al., 2003) or 5-S-GAD (Leem et al., 1996), although substantially higher than those of the antibacterial peptides, which range in the sub-micromolar to micromolar level (Hoffmann, 2003; Royet et al., 2005).

In conclusion, the results in this note indicate that NBAD and eventually other small molecules containing β-ala (Kano and Natori, 1984; Natori et al., 1999; Meylaers et al., 2003), which are products of one peculiar branch of insect catecholamine and dipeptide metabolisms, seem to participate in the third phase of the insect innate immune response, somehow supplementing the action of the antimicrobial peptides. Our results also represent a novel aspect of β-ala metabolism in insects and indicate that the expression of the *C. capitata niger* gene (not cloned yet) probably require three alternative regulatory mechanisms, one in brain and two in epidermis.

Acknowledgments

We thank Lorena Inzillo for sharing preliminary data from her Master's Thesis and Luciana Pujol-Lereis for a novel fly food. We are indebted to Jan Drijfhout (Leiden University, The Netherlands) and José M. Aguirre (University of Luján, Argentina), for the very helpful synthesis of NBAD derivatives. We also thank Beatriz Mendez (School of Sciences, University of Buenos Aires) for providing a *B. megaterium* strain. M.P. is a Fellow of the CONICET. L.Q.-A is full Professor at the University of Buenos Aires and Principal Researcher of the CONICET and of the Leloir Foundation. This work was supported by the University of Buenos Aires, the ANPCyT-SECyT-PICT-2003-351 and the CONICET.

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