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INVASIVE BEHAVIOUR AND DEPOLARIZATION EFFECT OF *PSEUDOMONAS FLUORESCENS* ON RAT CEREBELLAR GRANULE NEURONS

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Previous studies have shown that *Pseudomonas fluorescens* exerts cytotoxic effects on neurons and glial cells. In the present work, we investigated the time course effect of *Pseudomonas fluorescens* MF37 and of its lipopolysaccharide (LPS) on cultured rat cerebellar granule neurons. The kinetics of binding of *P. fluorescens* to cerebellar granule neurons is identical to that of cortical neurons but the binding index is lower, suggesting the presence of a reduced number of binding sites. As demonstrated by measurement of the concentration of nitrites in the culture medium, *P. fluorescens* induces a rapid stimulation (3 h) of the nitric oxide synthase (NOS) activity of the cells. In contrast, LPS extracted from *P. fluorescens* requires a long lag phase (24 h) before observation of an activation of NOS. Measurement of the resting membrane potential of granule neurons showed that within 3 h of incubation, there was no difference of effect between the action of *P. fluorescens* and that of its LPS endotoxin. Two complementary approaches allowed us to demonstrate that *P. fluorescens* MF37 presents a rapid invasive behaviour, suggesting a mobilisation of calcium in its early steps of action. The present study reveals that *P. fluorescens* induces the sequential activation of a constitutive calcium dependent NOS and that of an inducible NOS activated by LPS. Ours results also suggest that *P. fluorescens* cytotoxicity and invasion are not mutually exclusive events.

Key words: Cytotoxicity, Lipopolysaccharide, Patch-clamp, Invasion, *Pseudomonas fluorescens*

INTRODUCTION

Pseudomonas fluorescens, a bacterium closely related to the opportunistic pathogen *Pseudomonas aeruginosa* is considered to be as common as this species in the environment [1]. Few years ago, *P. fluorescens* was found to be responsible for 60% of the cases of post transfusion septicaemia in United Kingdom [2]. The involvement of *P. fluorescens* in infections of the central nervous system (CNS) in human remains controversial because of the difficulty in identifying the strains present in hospital [3]. However, this bacterium is known to cause local facial infections by using routes of penetration into the CNS identical to those of *P. aeruginosa* [3]. In addition, it is well established that *P. fluorescens* provokes CNS infections in fish [4] and express binding proteins for γ-aminobutyric acid, the main inhibitory neurotransmitter of the CNS [5, 6].

We have previously shown that *P. fluorescens* can bind to glial cells [7] and that its lipopolysaccharide (LPS) modulates potassium channels in target cells [8]. The dose-related effect of the LPS from *P. fluorescens* has been determined on glial cells and cortical neurons using a morphological approach [9]. Also, we have demonstrated that within 24 h, the LPS can induce the expression of a nitric oxide synthase (NOS) associated with apoptosis [9]. However, until now the time course of the cytotoxic effect of *P. fluorescens* on neurons has not been studied in details since this work requires a cell population to investigate the effect of both intact living bacteria and LPS on biochemical indicators

of cytotoxicity and on the plasma membrane potential.

In addition, since membrane ionic currents play a central role in the adhesion of bacteria to the target cells and on the internalisation activity [10], it appeared essential to correlate the cytotoxic activity of *P. fluorescens* to its binding and invasive potential.

In the present study, we selected a homogeneous neuronal cell line, cerebellar granule neurons, to compare the kinetics of the cytotoxic action of *P. fluorescens* MF37 and its LPS and the relative effect of the bacterium and its LPS endotoxin on the membrane currents. The results are discussed in regard to the activation of the different NOS expressed in neurons and to the invasive behaviour of the bacterial strain.

MATERIAL AND METHODS

Bacterial culture and LPS extraction

The strain MF37 of *Pseudomona fluorescens* is a natural rifampicin resistant mutant of the psychrotrophic strain MF0 isolated from crude milk [11]. *P. fluorescens* MF37 was grown at 28°C in Luria Bertani (LB). Just before the experiments bacteria in early stationary phase were harvested by centrifugation (6000 rpm, 4 min, 20°C) and resuspended in culture medium bathing neurons without antibiotics or antimycotics. The LPS from *P. fluorescens* was purified as previously described [12]. The degree of purification of the LPS and the absence of contaminant protein was controlled by capillary electrophoresis analysis of the extracted molecule following the technique of Picot *et al* [13]

Culture of neurons

Cerebellar granule neurons were obtained from 6-8 day old rats. The cerebellum was mechanically dispersed in culture medium consisting of DMEM/Ham's medium (3:1) supplemented with 10% foetal calf serum, 2 mM glutamine, 5 µg/ml insulin, 100 µg/ml transferrin, 20 µM putrescine, 1 mM sodium pyruvate, 30 nM sodium selenite, 25 mM KCl, 20 nM progesterone and containing 1% antibiotic-antimycotic solution. The suspension was filtered on a sterile 82 µm nylon filter to remove the remaining tissue fragments. Neurons were plated on glass coverslips at a density of 1.5×10^6 - 8×10^6 cells/ml after the experiments.

Adherence assays

For the adherence assays, the culture medium of neurons was replaced by medium without antibiotics and antimycotics, and the bacterial suspension was added at a concentration of 10^6 CFU/ml which roughly corresponds to that considered as a pathogenic dose [14]. The cells were fixed for 5 min in methanol and incubated in Coomassie blue 0.5% in methanol/water (V/V) and counterstained for 15 min in a Giemsa solution. Culture slides were rinsed 3 times for 5 min in methanol/water (1:1) and mounted under glass slides in phosphate buffer (PB; 0.1 M, pH 7.4) – glycerol (1:1). The binding index was determined by counting the number of bacteria adherent to the cytoplasmic membrane of the cells. Observations were done under an Axiovert S100 optical microscope (Zeiss, Oberkochen, Germany) equipped with an Olympus SC35 photographic system.

Determination of the nitric oxide synthase (NOS) activity

Nitrite ions (NO^{2-}), considered as representative of the activation of nitric 0oxide synthase (NOS) involved in the apoptotic process of neurons, were assayed using a technique derived from the Griess colorimetric reaction. The experiments were performed using neurons plated at high density (8 x 10^6 cells/ml) and after 7 days of culture in vitro (DIV=7). The assays were carried out using living bacteria (*P. fluorescens* 10^6 CFU/ml) and LPS from *P. fluorescens* (200ng/ml). Control studies were performed by the use of; i) bacteria incubated for the same period in culture medium for neurons but in the absence of the cells, and ii) an extraction buffer made following the same protocol used for the extraction of LPS but in the absence of bacteria. The intra- and inter-assay coefficients of variation were lower than 3 and 8% respectively

Measurement of the resting membrane potential (RMP) of neurons

Electrophysiological recordings were performed at room temperature (20-22°C) using the patch-clamp technique [15] in the whole-cell configuration in current-clamp mode. Recordings were made with a patch-clamp amplifier (Axopatch 200B, Axon Instruments Inc, USA). The culture medium was replaced by a bathing solution (pH = 7.4) containing 145 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose. Cells were allowed to adapt to the new medium for 15 min. The patch-pipette (3-5 MΩ, Harvard apparatus) was filled with a solution of 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA and 10 mM HEPES, (pH = 7.4). Data acquisition was performed with a computer-controlled interface using pclamp software version 8.1 (Axon Instruments, USA).

Tests of invasiveness

The invasive behaviour of *P. fluorescens* in cerebellar granule neurons was investigated using two complementary approaches, the microscopic acridine orange internalisation assay and the gentamicin survival assay.

In the acridine orange assay, which is based on the technique described by Goldner *et al* [16], neurons exposed to bacteria (10^6 CFU/ml) for 4 h were incubated with 0.01% acridine orange in Gey's solution for 45 s at room temperature. The samples were rinsed with fresh culture medium and then stained with 0.05% crystal violet in culture medium for 45 s. After rinsing, the slides were mounted with culture medium and immediately observed or photographed. Acridine orange enters freely into the neurone cells and stains both extracellular and intracellular bacteria. Crystal violet, which does not penetrate into the neurons, quenches the extracellular acridine fluorescence. Thus the only bacteria that can be visualised are those present inside the cells.

The gentamicin survival assay is a technique adapted from that used to quantify *Pseudomonas aeruginosa* invasion in epithelial cells [17]. Briefly, neurons were exposed to bacteria (10^6 CFU/ml) for 4 h in culture medium without antibiotics and antimycotics. The bacterial concentration was controlled by measurement of optical density and plating. At the end of the incubation period, cultured neurons were rinsed 3 times with fresh medium to remove free bacteria. Control cultures were immediately treated with 1 ml Triton X100

in PB (0.1% v/v). After plating and counting, the total number of bacteria present at the surface and inside the cells was determined. To determine the number of intracellular bacteria, after incubation with *P. fluorescens* and rinsing, the cultures were exposed to gentamicin (300µg/ml) for 1 h. The cultures were .then rinsed 3 times with 1 ml fresh culture medium to remove residual gentamicin and the cells were lysed with 1 ml Triton X100 in PB (0.1% v/v) before plating and counting. A blank was performed by incubating *P. fluorescens* MF37 for 1h with gentamicin (300µg/ml) to verify the effect of the antibiotic on the viability of the micro-organism. Colonies corresponding to living bacteria were counted after 2 days of culture at 28°C. Each measure was performed in triplicate.

Statistics

All data were calculated as mean ± S.E.M. Statistical analysis were evaluated by use of the unpaired Student's *t* test. The curves were fitted through polynomial regression equations using Sigma Plot V.

RESULTS

Adherence of P. fluorescens to cerebellar granule neurons and morphological effects

Cultured rat cerebellar granule neurons at day 7 were used. This culture period was previously found to correspond to the minimal duration allowing the cerebellar granule neurons to reach their mature state [18]. As illustrated in Fig. 1 A, adherence of bacteria to neurons occurred rapidly. After a 5-h period of incubation, the adsorption index reached 3.03 ± 0.14 bacteria/neuron. The adherence of bacteria to the plasma membrane was followed by marked morphological changes at the level of both the cell body and neurites. In the absence of bacteria, mature cerebellar granule neurons typically exhibited clear T-shapes and possessed several dendrites and thin axons with well defined cytoplasmic limits (Fig. 1 B). In contrast, in the presence of bacteria, a large number of vacuoles were observed in the cell body (Fig. 1 C). Moreover, a pronounced leakage of the cytoplasmic content was detected.

Kinetics of the stimulation of the NOS of cerebellar granule neurons by P. fluorescens and its LPS

The effect of *P. fluorescens* MF37 (10^6 CFU/ml) and of its lipopolysaccharide (LPS, 200 ng/ml) on the nitric oxide synthase (NOS) activity of cerebellar granule neurons was determined after 3, 12 and 24 hours of incubation. In the absence of treatment, the basal level of NO^{2-} measured in the medium was under the detection limit of the assay and remained undetectable after 3, 12 or 24 h of culture. A 3 hour incubation of neurons with *P. fluorescens* MF37 induced a rapid increase in the concentration of NO^{2-} that reached 22.3 ± 0.4 µg/ml (Fig. 2A). As indicated by the concentration of nitrite ions detected in the medium after 12 and 24 h of incubation (21.2 ± 0.9 µg/ml and 21.1 ± 1.2 µg/ml, respectively), the effect of living bacteria was already maximal after 3 h of incubation.

The kinetics of action of the LPS extracted from *P. fluorescens* was totally different (Fig. 2B). When cerebellar granule neurons were incubated for 3 or 12 h with LPS, the concentration of nitrite ions in the culture medium remained below the detection limit of the assay and apparently unchanged. It is only after 24 h of incubation that a rise in the concentration of NO^{2-} was observed and the value (11.1 ± 0.4 µg/ml) was only half of that measured with living bacteria.

Effect of P. fluorescens and LPS on the RMP of cerebellar granule neurons

The measures were performed on neurons exhibiting at least 2-3 adherent bacteria after 4 hours of incubation. The resting membrane potential of granule neurons exhibiting bacteria was significantly ($P < 0.001$) less negative (- 46.0 ± 4.7 mV, n=9) than in control neurons (- 63.7 ± 2 mV, n=12) (Fig. 3). The LPS extracted from *P. fluorescens* MF37 (200 ng/ml) provoked a membrane potential shift of the same range as that measured with the intact micro-organism (- 47.2 ± 4.8 mV, n=9) and this value was also significantly different ($P < 0.001$) from the control (n=9). In order to verify that the effect of the LPS was independent of artefacts the same measure was performed using the extraction buffer used to purify the endotoxin. Under these conditions, the resting membrane potential (-66.5 ± 2.4 mV, n=12) did not significantly differ from the control.

Invasive behaviour of P. fluorescens in cerebellar granule neurons

The microscopic acridine orange internalisation assay allowed us to visualise clearly stained bacteria in the cytoplasmic compartment of neurons (Fig. 4A). Counting of homologous fields showed that an average of 58% of granule neurons contained bacteria in their cytoplasm. This value is above the 5% accepted as the upper limit value to consider that a bacterium has an invasive behaviour [19]. The blank test performed for the gentamicin survival assay revealed that gentamicin 300µg/ml was capable of provoking a total destruction of *P. fluorescens* MF37 (10^6 CFU/ml) in 1 hour.

When cultured neurons were incubated with bacteria for 4 hours and then exposed for 1 h to gentamicin (300µg/ml), a significant number of viable bacteria were detected, suggesting that these micro-organisms were protected from gentamicin by the cytoplasmic membrane of the cells (Fig. 4 B). This population represented 32.6 ± 3.7 % of the total number of remaining bacteria associated with cultured neurons after a 4 h incubation and 3 rinsing steps. This percentage of invasive bacteria is in the same range as that obtained by the acridine orange internalisation assay.

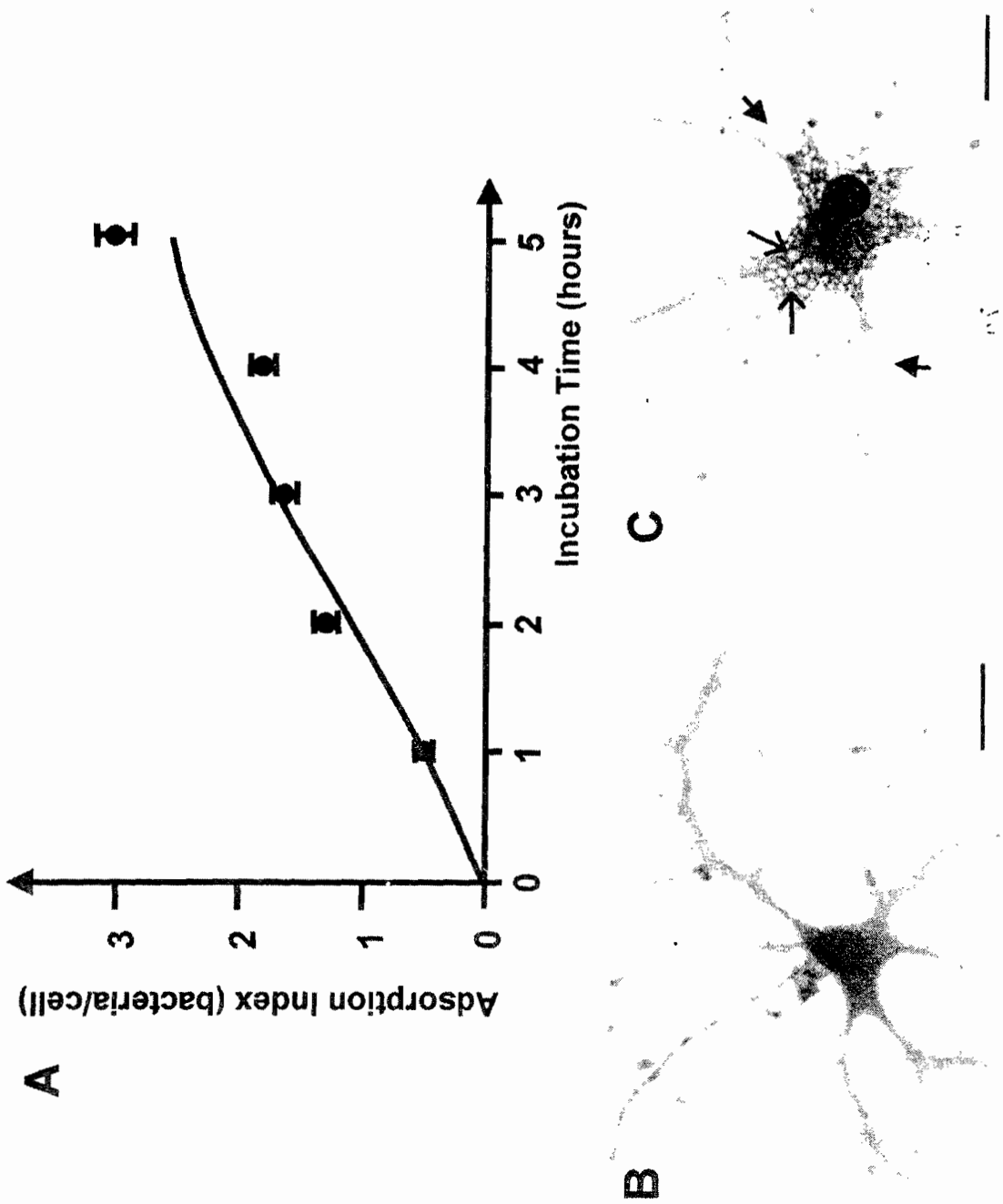


Fig. 1: Effects of *Pseudomonas fluorescens* MF37 (10^6 CFU/ml) on cerebellar granule neurons at day 7. (A) Time-course curve of the adherence of the bacteria. Each point corresponds to the mean number of adherent bacteria/neuron (± SEM) and was determined over a minimum of 50 neurons. (B-C) Photomicrographs of cultured cerebellar granule neurons in the absence (B) or presence (C) of bacteria after 5h exposure. Note that neurites have lost their cytoplasmic content (⟶) while the soma is markedly vacuolated (⟶).

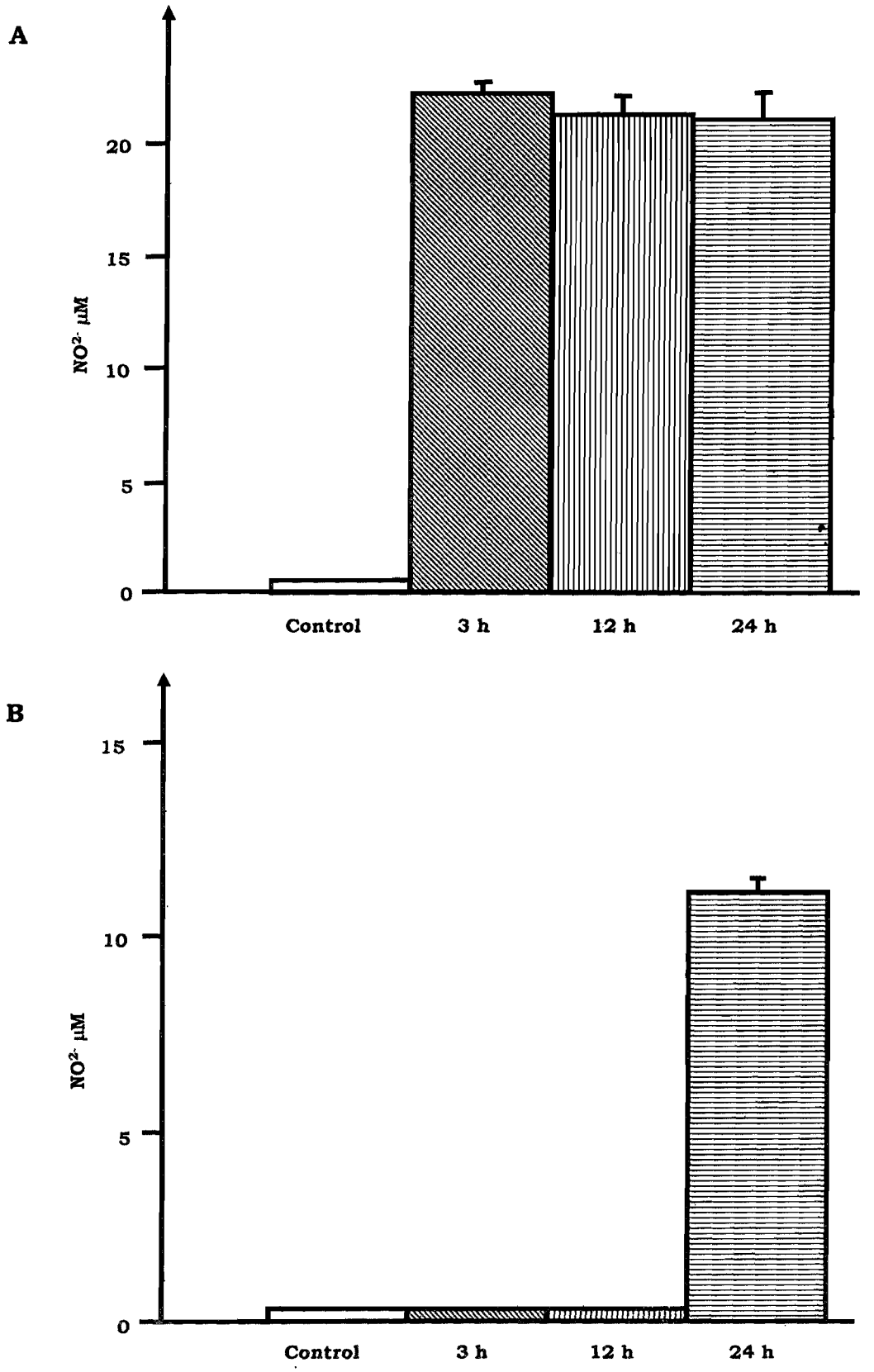


Fig. 2 Effects of *Pseudomonas florescens* and its LPS on NOS activity of cerebellar granule neurons. (A) Effects of the living bacteria (10^6 CFU/ml) on concentration of NO^{2-} measured in culture medium after 3, 12, and 24 hours of incubation. Control studies were performed to show absence of NOS activity in *P. florescens* MF 37 in the experimental conditions. (B) Effects of the LPS extracted from *P. florescens* (200ng/ml) on the concentration of NO^{2-} measured in culture medium after 3, 12 and 24 hours of incubation

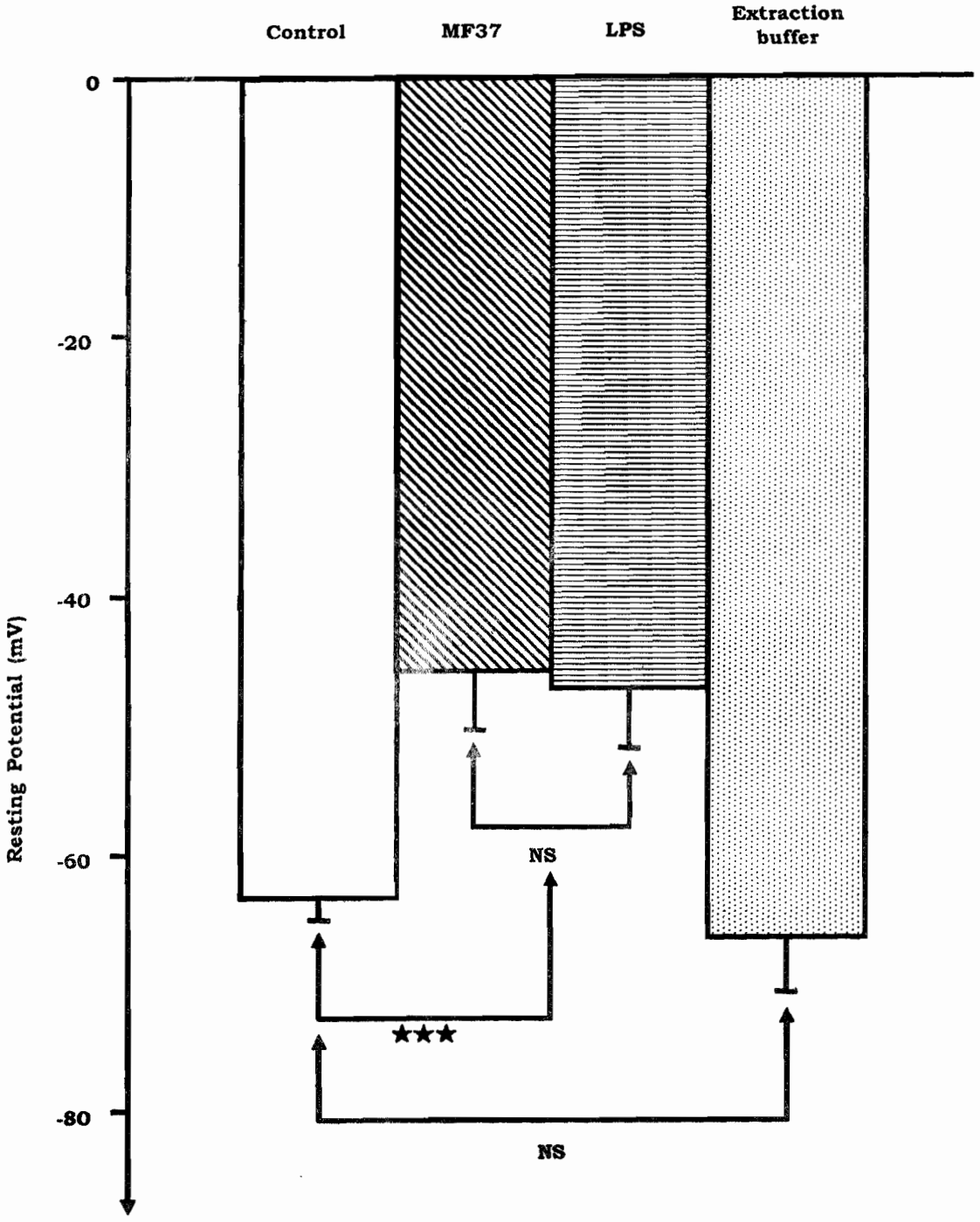
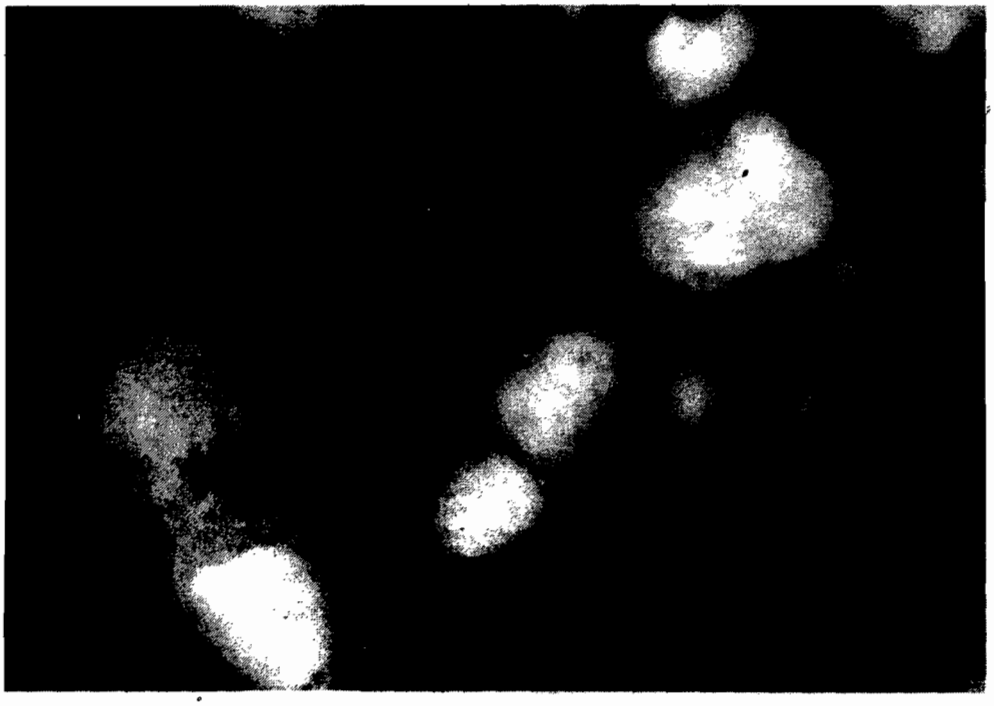


Fig. 3. Effects of *Pseudomonas florescens* MF 37 and its LPS on the resting membrane potential (RMP) of cultured cerebellar granule neurons at day 7. The histograms represent the level of the RMP in control neurons (Left bar), neurons incubated for 4 h with *P. fluorescens* 10^6 CFU/ml and exhibiting at least 1-2 adherent bacteria (Second bar), neurons incubated for 3 h with 200 ng/ml LPS (Third bar) or with the extraction buffer employed for the purification of LPS (Fourth bar). Each bar corresponds to the mean value (± SEM) of 9 measurements from 3 independent experiments.



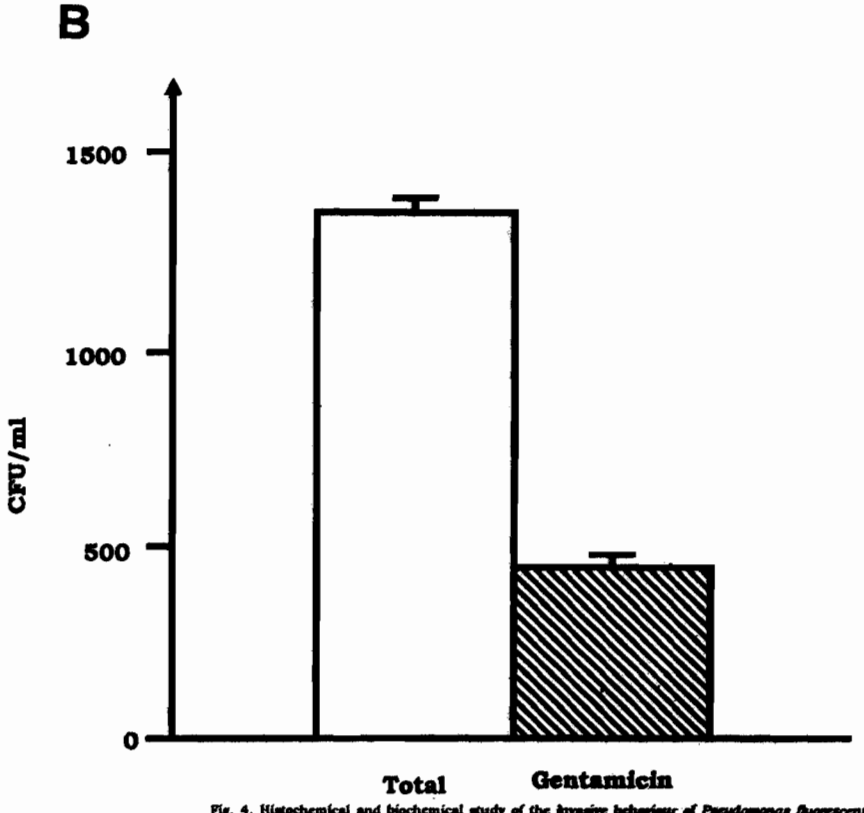


Fig. 4. Histochemical and biochemical study of the invasive behaviour of *Pseudomonas fluorescens* MF37 on cerebellar granule neurons. (A) Photomicrograph showing bacteria labelled by acridine orange in the cytoplasm of granule neurons. The counting of homologous fields showed that a mean of 58% of granule neurons contained labelled intracellular bacteria. (B) Gentamicin survival assay of P. fluorescens on cerebellar granule neurons. The left bar illustrates the total number of living bacteria (determined as CFU/ml) on or into granule neurons after 4 h of incubation. The right bar illustrates the number of living bacteria (determined as CFU/ml) present into granule neurons after 4 h of incubation and destruction of extracellular bacteria by gentamicin (300μg/ml). A control study was realised to verify that gentamicin (300μg/ml) induces a total destruction of non-protected P. fluorescens MF37 in the experimental conditions.

DISCUSSION

Since it is postulated that the virulence factors produced by *Pseudomonas* are essentially released in response to a cascade of events initiated by the binding of the bacterium to its host [1], a first step of our study was required to determine the kinetics of binding of *P. fluorescens* on cerebellar granule neurons, a cell type on which these bacteria have not been investigated before. The profile of the binding curve of *P. fluorescens* on cerebellar granule neurons was similar to that previously observed on cortical neurons [7]. In contrast, the binding index of *P. fluorescens* on cerebellar granule neurons was limited to a mean of 3 bacteria per cells whereas it reached 12 bacteria per cell in cortical neurons [7]

The heterogeneity of the cell types present in cortical neurons in primary culture is much higher than in cerebellar granule neurons cultures and the difference of binding index may be ascribed to the presence in the population of cortical neurons of cell lines presenting a very high affinity for bacteria. However, the limited variations of the statistical values in both studies is not in favour of this hypothesis and, since the size of cortical and cerebellar neurons in primary culture is in the same range, we can presume that the number of binding sites for bacteria in cerebellar neurons is lower than in cortical neurons. In spite of these differences, the morphological effects of *P. fluorescens* are similar in cerebellar and cortical neurons. Within 5 hours of incubation with the bacteria, cerebellar granule neurons presented vacuolar inclusions and nuclear aggregations typical of apoptosis [20]. Thus, cerebellar granule neurons appeared as a valuable representative model for the study of the time course effect of *P. fluorescens* and its LPS.

Until now, the effect of *P. fluorescens* on the formation of NO^{2-} in the culture medium of eukaryotic cells, which results from the activation of NOS, was only determined after 24 hours of exposure to the bacteria [13]. The present study revealed that the activation of NOS in granule neurons is very rapid since 3 hours incubation with *P. fluorescens* is sufficient to reach the maximum response. As clearly indicated by experiments of brain ischemia in animal models, the stimulation of NOS is an early event in the apoptotic process of neurons [21] and the observation of a rapid rise of the concentration of nitrites (NO^{2-}) following exposure of neurons to bacteria is in agreement with our previous observations [7].

A striking result was the delay in response of neurons to the LPS extracted from *P. fluorescens*. In addition, even after 24 h of incubation, when a rapid increase in the production of nitrites was observed, the level of NO^{2-} measured in the medium was only half of that obtained using the living bacteria. The LPS was used at a concentration (200ng/ml) corresponding to that potentially released by bacteria at the concentration employed in the present study (10^6 CFU/ml) [9]. Even though LPS probably plays a major role in the cytotoxic effect of *P. fluorescens* MF37, the present results suggest that at least another virulence factor is essential in the very early effects of the bacteria. In cerebellar granule neurons, two types of NOS have been identified, a constitutive type 1 NOS (nNOS), the activity

of which depends on calcium/calmodulin, and an inducible or type 2 NOS (iNOS) [22]. Rodrigo *et al* [23] have shown that an increase in the level of expression of iNOS can occur within 2 to 4 hours after stimulation. A concomitant activation of nNOS can also take place. However, a delay of 3 hours is short to provoke a maximal induction of iNOS, the massive accumulation of its metabolite (NO) and its spontaneous conversion into nitrites, as detected in the medium. Thus, it appears that the rise of NO observed with intact bacteria within 3 hours may be essentially ascribed to a direct activation of nNOS whereas the action of LPS, characterised by a long lag phase, could correspond to the activation of iNOS.

The activity of nNOS is controlled by transient increases of calcium resulting from the opening of voltage dependent calcium channels activated by membrane depolarisation [22]. The resting membrane potential of cerebellar granule neurons was studied using matured cells at 7 DIV since, as previously observed by Shibata *et al* 18], we observed that the resting membrane potential of the cells, initially less negative (– 41 ± 1.2 mV (n=12) at days 2-3), stabilised from day 7 to a mean value of - 63.7 ± 2 mV (n=9). The resting membrane potential of neurons was measured in cells exhibiting 2 to 3 adherent bacteria and in cells exposed for 3 hours to LPS. Living bacteria and LPS both induced a significant shift of the resting membrane potential to less negative values, – 46.0 ± 4.7 mV and - 47.2 ± 4.8 mV (n=9), respectively ($P < 0.001$). These results were not biased by compounds present in the extraction buffer used to purify LPS as demonstrated by control studies. The absence of difference between the electrophysiological effect of the living bacteria and its endotoxin demonstrates that LPS is the major virulence factor responsible for membrane depolarization in cerebellar granule neurons. The absence of significant difference between the effect of bacteria and LPS in these experiments also indicates that the rise of NO measured after 3 hours using living cells is independent of LPS and probably involves other virulence factor(s).

The membrane depolarization observed in the present study is in agreement with our previous works, showing that in cerebellar granule neurons, LPS provokes the reduction of two of the major voltage-dependent potassium currents [8]. It is known that in neurons depolarization triggers the activation of N-methyl-D-aspartic (NMDA) receptors coupled to calcium influx and to the activation of nNOS [24]. Prolonged activation of NMDA receptors leads to apoptosis [25]. Thus, the present results suggest that the bacterium and its LPS provoke the biosynthesis of NO by two pathways resulting in cell death.

The hypothesis that *P. fluorescens* rapidly activates nNOS presumes that the membrane depolarization activates local calcium influx. This presumption is supported by the morphological changes provoked by the bacteria. Calcium influx plays a central role in cytoskeletal rearrangements involved in cell binding and internalisation of bacteria [10]. In the present study, we demonstrate using two different and complementary approaches that, in addition to binding to cerebellar granule neurons, *P. fluorescens* MF37 exhibit an invasive behaviour that can be

observed within 4 hours of contact, before any effect of LPS on NOS. As indicated from the gentamicin assay, *P. fluorescens* MF37 survived through the internalisation process, but since *P. fluorescens* MF37 is a psychrotrophic strain, it is impossible to determine if the bacteria were able to grow, in the intracellular compartment.

Nonetheless, since they suggest that rapidly after binding of *P. fluorescens* to its target cell, local changes in calcium concentrations coupled to internalisation occur, these results support the hypothesis of an early stimulation of the calcium dependent nNOS. Further experiments should be performed to characterise the calcium channels involved in this process, but since the pharmacological agents used to selectively block ionic channels could also affect the bacterial physiology, this study deserves a complex electrophysiological approach. On the other hand, it is important to note that studies performed with *P. aeruginosa* have shown that in epithelial cells, invasion and cytotoxicity are independent and mutually exclusive events [17].

Thus, the situation of *P. fluorescens* MF37 appears more ambiguous since in addition to presenting a significant, and occasionally very active cytotoxic activity when grown at low temperature [13], this bacterium also exhibit an invasive behaviour. These results are in agreement with clinical observations showing that whereas *P. fluorescens* is generally a low virulence micro-organisms, some strains can behave as life threatening opportunistic pathogens [3].

Taken together, these results demonstrate that *P. fluorescens* can sequentially activate constitutive and inducible NOS expressed in cerebellar granule neurons. We have shown that LPS is essentially involved in a late induction of iNOS whereas other virulence factors are possibly responsible for the early effects of the bacterium and could control its invasive behaviour.

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REFERENCES

1. Hardalo C, Edberg SC. *Pseudomonas aeruginosa*: assessment of risk from drinking water. *Crit. Rev. Microbiol.* 1997; **23**: 47-75
2. Puckett A, Davison G, Entwistle CC, Barbara JA. Post transfusion septicaemia 1980-1989: importance of donor arm cleaning. *J. Clin. Pathol.* 1992; **45**: 155-157
3. Feuilloley MGJ, Mezghani-Abdelmoula S, Picot L, *et al.* Involvement of *Pseudomonas* and related species in central nervous system infections. *Recent Res. Dev. Microbiol.* 2003; **7**: 55-71
4. Mastan SA, Qureshi TA. Role of bacteria in the epizootic ulcerative syndrome (EUS) of fishes. *J. Environ. Biol.* 2001; **22**: 187-192
5. Tunnicliff G. Inhibition of 4-aminobutyrate aminotransferase from *Pseudomonas fluorescens* by ATP. *Biochem. Mol. Biol. Int.* 1993; **31**: 41-47
6. Guthrie GD, Nicholson-Guthrie CS, Leary HL. A bacterial high-affinity GABA binding protein: isolation and characterization. *Biochem. Biophys. Res. Commun.* 2000; **268**: 65-68
7. Picot L, Abdelmoula S, Merieau A, *et al.* *Pseudomonas fluorescens* as a pathogen: adherence to nerve cells. *Microb. Infect.* 2001; **3**: 985-995
8. Mezghani-Abdelmoula S, Chevalier S, Lesouhaitier O, Orange N, Feuilloley MGJ, Cazin L. *Pseudomonas fluorescens* lipopolysaccharide inhibits both delayed rectifier and transient A-type K^+ channels of cultured rat cerebellar granule neurons. *Brain Res.* 2003; **983**: 185-192

9. Picot L, Chevalier S, Mezghani-Abdelmoula S, *et al.* Cytotoxic effects of the lipopolysaccharide from *Pseudomonas fluorescens* on neurons and glial cells. *Microbial. Pathogen.* 2003; **35**: 95-106
10. Tran Van Nhieu G, Clair C, Grompone G, Sansonetti P. Calcium signalling during cell interactions with bacterial pathogens. *Biol. Cell.* 2004; **96**: 93-101
11. Burini JF, Gügi B, Merieau A, Leriche F, Guespin-Michel J. Genetic studies of a thermoregulated gene in the psychrotrophic bacterium *Pseudomonas fluorescens. Res. Microbiol.* 1994; **150**: 447-456
12. Darveau RP, Hancock PE. Procedure for isolation of bacterial lipopolysaccharides from smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* 1983; **155**: 831-838
13. Picot L, Chevalier S, Mezghani-Abdelmoula S, *et al.* Regulation of the cytotoxic effects of *Pseudomonas fluorescens* by growth temperature. *Res. Microbiol.* 2004; **155**: 39-46
14. George SE, Nelson GM, Kohan MJ, Brooks LR, Boyd C. Colonization and clearance of environmental microbial agents upon intranasal exposure of strain C3H/HeJ mice. *J. Toxicol. Environ. Hlth.*1999; **56**: 419-431
15. Hamill OP, Huguenard JR, Prince DA. Patch-clamp studies of voltage-gated currents in identified neurons of the rat cerebellar cortex. *Cerebr. Cortex* 1991; **1**: 48-61
16. Goldner M, Farkas-Himsley H, Kormendy A, Skinner M. Bacterial phagocytosis monitored by fluorescens and extracellular quenching: ingestion and intracellular killing. *Lab. Med.* 1983; **14**: 291-294
17. Evans DJ, Frank DW, Finck-Barbacon V, Wu C, Fleiszig SM. *Pseudomonas aeruginosa* invasion and cytotoxicity an independent events, both of which involve protein tyrosine kinase activity. *Infect. Immun.* 1998; **66**: 1453-1459
18. Shibata R, Nakahira K, Shibasaki K, Wakazono Y, Imoto K, Ikenaka K. A-type K^+ current mediated by the Kv4 channel regulates the generation of action potential in developing cerebellar granule cells. *J. Neuroscience* 2000; **20**: 4145-4155
19. Edberg SC, Kops S, Kontnick C, Escarzaga M. Analysis of cytotoxicity and invasiveness of heterotrophic plate count bacteria (HPC) isolated from drinking water on blood media. *J. Appl. Microbiol.* 1997; **82**: 455-461
20. Häcker G. The morphology of apoptosis. *Cell Tissue Res.* 2000; **301**: 5-17
21. Mattson MP, Culmsee C, Fan Yu Z. Apoptotic and anti-apoptotic mechanisms in stroke. *Cell Tissue Res.* 2000; **301**: 173-187
22. Kolb JP. Pro- and anti-apoptotic role of nitric oxide (NO). *C.R. Acad. Sci. Paris* 2001; **324**: 413-424
23. Rodrigo J, Alonso D, Fernandez AP, *et al.* Neuronal and inducible nitric oxide synthase expression and protein nitration in rat cerebellum after oxygen and glucose deprivation. *Brain Res.* 2001; **909**: 20-45
24. Rameau GA, Chiu LY, Ziff EB. NMDA receptor regulation of nNOS phosphorylation and induction of neuron death. *Neurobiology of Aging* 2003; **24**: 1123-1133
25. Khodorov B. Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurons. *Progr. Biophys. Mol. Biol.* 2004 (In Press).

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