INVOLVEMENT OF THE MITOGEN-ACTIVATED PROTEIN (MAP) KINASE SIGNALLING PATHWAY IN HOST CELL INVASION BY *TOXOPLASMA GONDII*

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Summary :

Little is known about signalling in *Toxoplasma gondii*, but it is likely that protein kinases might play a key role in the parasite proliferation, differentiation and probably invasion. We previously characterized Mitogen-Activated Protein (MAP) kinases in *T. gondii* lysates. In this study, cultured cells were tested for their susceptibility to *Toxoplasma gondii* infection after tachyzoite pretreatment with drugs interfering with MAP kinase activation pathways. Protein kinases inhibitors, i.e. genistein, RO31-8220 and PD098059, reduced tachyzoite infectivity by 38 ± 4.5 %, 85.5 ± 9 % and 56 ± 10 %, respectively. Conversely, protein kinases activators, i.e. bombesin and PMA, markedly increased infectivity (by 202 ± 37 % and 258 ± 14 %, respectively). These results suggest that signalling pathways involving PKC and MAP kinases play a role in host cell invasion by *Toxoplasma*.

KEY WORDS : *Toxoplasma gondii*, protein kinases, MAP kinases, cell culture, intracellular signalling, cell invasion.

Résumé : RÔLE POSSIBLE D'UNE TRANSDUCTION DU SIGNAL VIA LES "MITOGEN-ACTIVATED PROTEIN" (MAP) KINASES DANS L'INVASION DE LA CELLULE PAR *TOXOPLASMA GONDII*

Il existe peu de données sur les voies de signalisation chez Toxoplasma gondii, *mais il est probable que des protéines kinases jouent un rôle dans la croissance, la différenciation, mais aussi l'invasion du toxoplasme. Nous avons précédemment caractérisé des "mitogen-activated protein" (MAP) kinases dans le toxoplasme. Dans cette étude, nous montrons que le pré-traitement des tachyzoïtes par des activateurs (bombésine, PMA) ou des inhibiteurs (génistéine, RO31-8220, PD098059) de protéine kinases intervenant dans la voie d'activation des MAP kinases, entraîne une augmentation ou une diminution de leur pouvoir infectieux pour des cellules 3T3 en culture, respectivement. Ces résultats sont en faveur d'un rôle des voies de signalisation impliquant des protéines kinases C et des MAP kinases dans l'invasion de la cellule par* T. gondii.

MOTS CLÉS : Toxoplasma gondii, *proteine kinases, MAP kinases, signalisation cellulaire, invasion, culture cellulaire.*

INTRODUCTION

Protein phosphorylation or dephosphorylation is involved in the enzymatic cascades which transduce signals in eukaryotic cells. Signal transduction plays important roles in cell differentiation and proliferation, and in the regulation of metabolic pathways. Phosphatases and kinases have been widely studied in eukaryotic cells, but few data are available on their presence and function in protozoan parasites. An important role of protein kinases and signal transduction has been demonstrated in some protozoan parasites, including *Leishmania* (Becker & Jaffe, 1997), *Trypanosoma cruzi* (Ogueta *et al.*, 1996), *Entamoeba histolytica* (Perez *et al.*, 1996), *Giardia duodenalis* (Chen *et al.*, 1996), and *Plasmodium falciparum* (Dluzevski & Garcia, 1996). Indeed, it has been documented that the phosphorylation pattern of *Leishmania* and other kinetoplastids varies throughout their life cycle, and may play a key role in parasite survival, differentiation, and cell invasion (Valentine & Carter, 1993; Hermoso & Jaffe, 1993 ; Dell & Engell, 1994; Parsons *et al.*, 1995; Wiese, 1998). Protein kinase inhibitors reduce the infectivity and growth of *Leishmania* and *Trypanosoma cruzi* (Vieira *et al.*, 1994; Becker & Jaffe, 1997). Several protein kinases genes have been identified in *Toxoplasma*, but their functions remain to be determined (Ng *et al.*, 1995; Ng *et al.*, 1997; Wastling & Kinnaird, 1998).

We previously demonstrated the presence of mitogen-activated protein (MAP) kinase activity in *T. gondii* and putatively identified two MAP kinases homologues of the ERK (extracellular signal-regulated kinases) family (Roisin *et al.*, 2000). Gomez-Marin *et al.* (1998) have also characterized mitogen-activated protein (MAP) kinase activity in *T. gondii*, that was decreased by INF-γ. As cell invasion and parasite replication within the host cell probably involve protein kinases, we examined the impact of protein kinases inhibitors and activators on *Toxoplasma* infectivity, focusing on compounds acting at different steps of MAP kinase activation pathways. Activation of MAP kinases (also known as extracellular signal-regulated kinases, or ERK) is one of the most

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rapid cellular responses to various external stimuli, leading to differentiation, growth and other cellular functions (Blenis, 1993). MAP kinase pathway activation occurs through G protein-coupled receptors, calcium channel-coupled receptors, and tyrosine kinase receptors (Treisman, 1996), and is highly dependent on the intracellular calcium concentration and protein kinase C (PKC) activation (Cobb & Goldsmith, 1995; Robinson & Cobb, 1997). In previous experiments, we observed that MAP kinase activity detected in toxoplasma lysates was stimulated by calcium or through PKC activation (Roisin *et al.*, 1999). We therefore tested *Toxoplasma* infectivity and growth in cultured cells after tachyzoite pretreatment with several compounds: 1) the tyrosine kinase inhibitor genistein, 2) the PKC inhibitor bisindoylmaleimide or RO31-8220 (Chao *et al.*, 1992), 3) the MAP kinase kinase (or MEK) inhibitor PD098059 (Alessi *et al.*, 1995), 4) the PKC activator phorbol-myristyl acetate (PMA) (18), and 5) the PKC and MAP kinase activator bombesin (Pang *et al.*, 1993). Our results point to a role of MAP kinases and PKC in host cell invasion by *T. gondii*.

MATERIALS AND METHODS

CELL CULTURES

NIH-3T3 cells were used for invasion experiments. Briefly, cells were cultured on glass coverslips in 12-well culture plates (70,000 cells per well) overnight at 37° C with 5 % CO_2, in 1 ml Dulbecco's Eagle modified medium (DMEM) containing 10 % fetal calf serum (FCS), 5 IU/ml penicillin and 5 Ug/ml streptomycin. One hour before invasion experiments with *Toxoplasma* tachyzoites, the medium was removed and replaced with DMEM containing 5 % FCS.

PARASITES

The RH strain of *Toxoplasma gondii* was maintained by serial passages in IOPS-OF1 Swiss mice (Iffa Credo, France). Tachyzoites were harvested from peritoneal fluid of mice infected four days earlier. The fluid was centrifuged for 10 min at 1000 g. The pellet was washed and resuspended in phosphate-buffered saline (PBS) containing 10 IU/ml penicillin and 10 U/ml strepto-

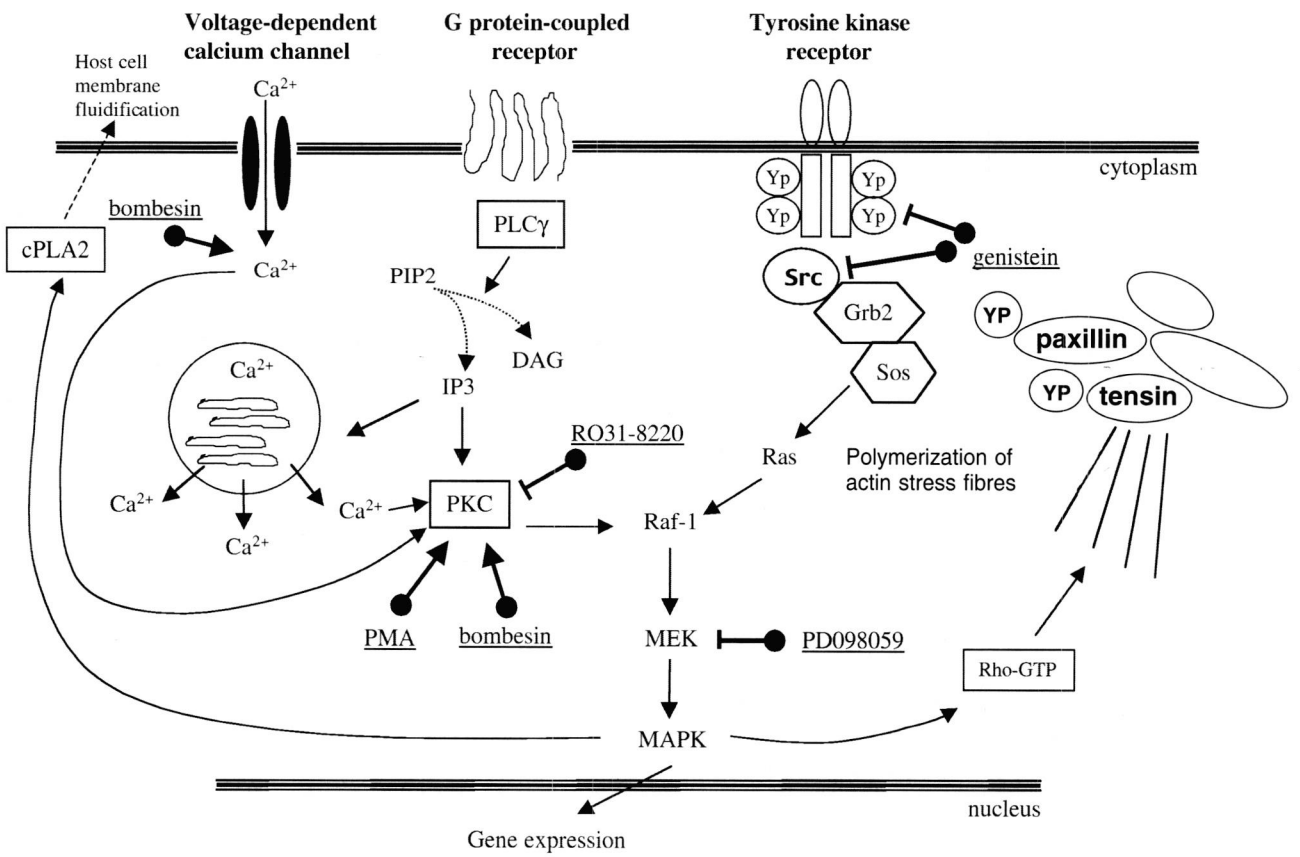

Fig. 1. – Biochemical targets of the different kinase activators (⟶) or inhibitors (⟞) used. Three main signalling pathways are concerned: the tyrosine kinase receptors pathway, the G protein-coupled receptors pathway, and the voltage-dependent calcium channels pathway. Abbreviations used: PLCγ: phospholipase Cγ; YP: tyrosine phosphorylated residue; PIP2: phosphatidylinositol-4,5-bisphosphate; DAG: diacylglycerol; IP3: inositol trisphosphate; PKC: protein kinase C; MEK: MAP kinase kinase.

mycin. Tachyzoites were counted and their viability was assessed by trypan blue exclusion method. Parasites were 95 % viable.

TREATMENT OF TACHYZOITES AND INVASION EXPERIMENTS

The site of action in the MAP kinase activation pathway, of the different compounds used, is reported in Figure 1. For drug treatment, tachyzoites (5×10^6) were resuspended in a final volume of 50 ml containing various concentrations of drugs, diluted either in DMEM (genistein (Alexis), RO31-8220 (Alexis, bombesin (Sigma), and PMA [Sigma]), or in 7 % bovine serum albumin (PD098059 (Alexis]), and incubated for 15 min (all drugs except PD 098059) or 30 min (PMA and PD098059) at 37° C with 5 % CO_2. Untreated control tachyzoites were incubated for the same times at 37° C. For invasion experiments, 10^6 treated or untreated tachyzoites were inoculated per well (parasite:cell ratio 10:1). The volume of inoculum was adjusted so that the final dilution of the drug in the well was at least 1:100 of the initial concentration, thus rending unlikely a direct effect on cell cultures. The absence of any effect on cells of all the drugs applied at such concentrations had been checked previously. After two hours of incubation at 37° C, *Toxoplasma* tachyzoites were removed and cells were washed extensively with DMEM then reincubated overnight. The cells were fixed for 30 min in 4 % paraformaldehyde then washed and kept in PBS until immunofluorescence staining. Each drug experiment was repeated at least three times.

QUANTIFICATION OF HOST CELL INVASION

• Immunocytofluorescence staining and microscopic quantification

Host cell invasion was quantified by counting after immunocytofluorescence (IF) staining of cell coverslips, using specific anti-*Toxoplasma* antibodies, as previously described (Creuzet *et al.*, 1998). Coverslips were also incubated for 30 min with phalloidin (diluted 1:200 in PBS containing 2 % FCS and 0.25 % Triton X-100) in order to visualize precisely cell structure. Coverslips were mounted in Vectashield (Vector, Biosys) and examined under an epifluorescence microscope (Nikon). At least 1000 cells were counted per coverslip. The percentage of infected cells and the mean number of tachyzoites per infected cell were recorded. The data allow a precise quantification of the efficacy of invasion. Results are expressed as the relative reduction or relative increase in cell infection induced by tachyzoite treatment, relative to untreated tachyzoites. Results are presented as mean (± SE) infection rates determined in three independent experiments.

• ^{3}H-uracil incorporation assay

In some experiments, cell infection was also determined by ^{3}H-uracil incorporation assay, as previously described (Pfefferkorn & Pfefferkorn, 1977). In this latter case, identical numbers of cultured cells (10^4 cells/cm^2) were seeded on coverslips. Cells were infected with 2×10^5 treated or untreated parasites per well for 2 h, then washed and incubated for the next 24 h in labeling medium containing dialyzed FCS (3 %) and (5-6)-[^{3}H]-uracil (2 µCi/ml, Amersham). The medium was then discarded, cells were lysed in PBS containing 1 % sodium dodecyl sulfate and 1 mM uracil, and protein was precipitated with trichloracetic acid (10 % final concentration). Precipitates were washed on GF/C filters (Whatmann), and the radioactivity on filters was counted in a scintillation counter. This technique allows to quantify the parasite mutiplication, and thus reflects roughly the efficacy of invasion. Results are expressed as the ratio incorporation rate of control tachyzoites : incorporation rate of treated tachyzoites. The results are presented as the means (± SE) of at least three independent experiments.

RESULTS

EFFECT OF PROTEIN KINASE INHIBITORS ON CELL INVASION AND PARASITE GROWTH

Toxoplasma tachyzoites incubated with the PKC-inhibitor RO31-8220 showed reduced infectivity for 3T3 cells, in a dose-dependent manner (Fig. 2A). The percentage of infected cells counted after IF staining decreased by 55 ± 8 % (*t* Student test, $p < 0.01$) when parasites were submitted to 100 µM RO31-8220 for 15 min before cell contact. In addition, the number of parasites per infected cell, counted after overnight incubation of infected cells, showed a marked decrease, from 6 (± 0.8) tachyzoites/cell (control) to 3.5 (± 0.6) (100 µM RO31-8220), suggesting reduced parasite replication (not shown). The assessment of parasite growth by [^{3}H]-uracil incorporation supported these data by demonstrating a marked dose-dependent fall in radioactivity incorporation, to 6.4 ± 2.1 % of control at 100 µM (*t* Student test, $p < 0.01$), and reflecting both reduced infectivity and reduced parasite replication (Table I).

Parasite incubation with genistein, a tyrosine kinase inhibitor, reduced infectivity by 38 ± 4.5 % compared to the control (*t* Student test, $p < 0.01$), but only at a high concentration (Fig. 2B). Similarly, [^{3}H]-uracil incorporation showed a slight decrease (Table I). Parasite replication was not significantly altered, as the mean number of parasites per infected cell was not different from the control value (not shown).

Drug (15 min incubation)		Drug concentration	Uracil incorporation (percentage of control, mean ± SE)
Protein kinase inhibitors	RO31-8220	5 µM	71 ± 8 %
		10 µM	68 ± 12.3 %
		50 µM	58 ± 9.2 %
		100 µM	6.4 ± 2.1 %
	Genistein	1 µM	86 ± 4 %
		10 µM	89.6 ± 2 %
		20 µM	88 ± 2 %
		50 µM	84 ± 2 %
MAP kinase and PKC activators	Bombesin	1 nM	134.5 ± 12.3 %
		5 nM	136.7 ± 13.6 %
		10 nM	150 ± 7.9 %
		50 nM	143.5 ± 6.5 %
		100 nM	133.5 ± 1.5 %
	PMA	100 nM	169.2 ± 13.8 %
		500 nM	108.5 ± 7.5 %
		1000 nM	116.5 ± 9 %

Data are means ± SE of three independent experiments.

Table I. – Effects of protein kinase inhibitors and activators on the intracellular development of *T. gondii in vitro*. Quantification by [^{3}H]-uracil incorporation after 24 h culture.

By contrast, parasite preincubation for 30 min with PD098059, a MEK inhibitor, led to a marked decrease in *Toxoplasma* infectivity (– 50 % at 10 µM) (Fig. 2C) (*t* Student test, $p < 0.001$).

Effect of kinases activators on parasite infectivity and growth

Tachyzoite preincubation for 15 min with bombesin resulted in a marked increase in *Toxoplasma* infectivity, which reached 202.6 ± 37 % of the control value at 50 nM (*t* Student test, $p < 0.001$) (Fig. 3A). Quantification of parasite replication by [^{3}H]-uracil incorporation yielded similar but lower results (Table I).
Similarly, the PKC activator PMA led to an increase in infectivity, which reached 258 ± 14 % of control at 500 nM after 30 min of stimulation (t Student test, $p < 0.001$) (Fig. 3B). PMA also increased infectivity after 15 min of stimulation, but to a lesser degree (+ 150 %). Whatever the incubation time, higher PMA concentrations had no effect on infectivity. This type of dose response is commonly observed with PMA, the effect of which is both dose- and time-dependent. [^{3}H]-uracil gave similar results (Table I).

DISCUSSION

It is likely that protein kinases play a key role in many cellular process in *Toxoplasma*, as well as in other eukaryotic cells. In this study we examined several signalling pathways leading to MAP kinase activation, by pre-treating parasites with several protein kinases inhibitors and protein kinases activators. The quantification of host cell invasion was assessed

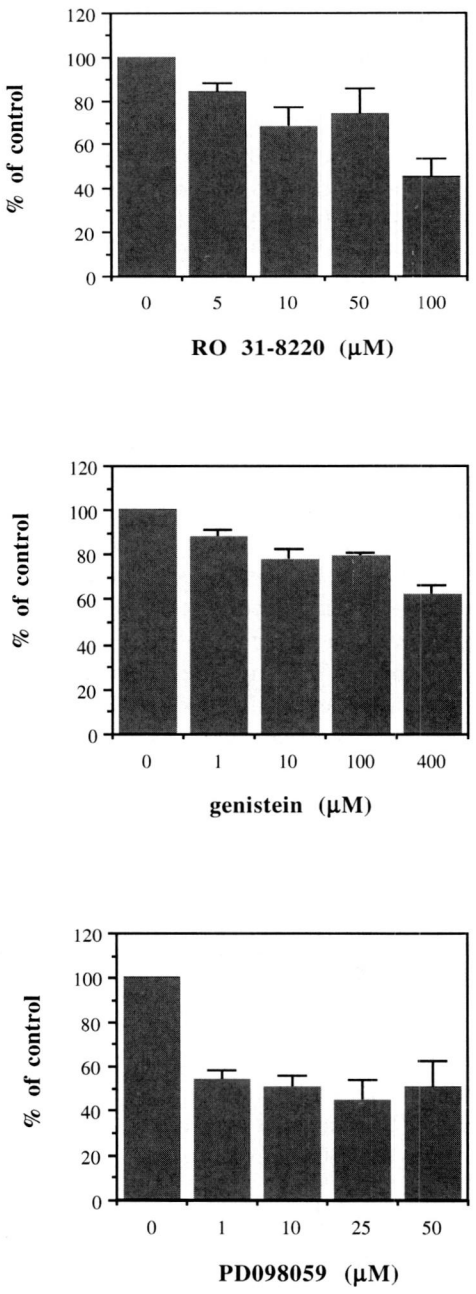

Fig. 2. – Effects of protein kinase inhibitors on *Toxoplasma* infectivity.

Toxoplasma tachyzoites were preincubated with increasing concentrations of RO31-8220 for 15 min (A), genistein for 15 min (B), or PD098059 for 30 min (C).

The efficacy of invasion was evaluated by microscopic counting of the percentage of infected cells. Results are expressed as relative percentages, corresponding to the relative decrease of cell infection, compared to control values (cell infection by untreated tachyzoites, i.e., 25.5 ± 6.9 % in (A), 20 ± 2.5 % in (B) and 34.5 ± 4.5 % in (C)), arbitrarily brought to 100 %. Data are the means ± SE of three independent experiments.

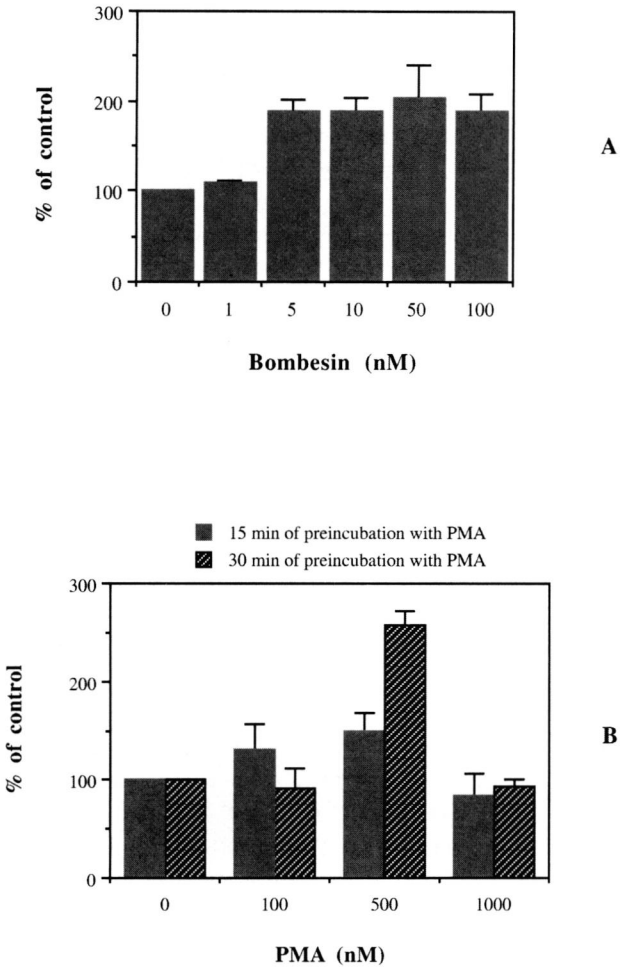

Fig. 3. – Effects of MAP kinase and PKC activators on *Toxoplasma* infectivity.

Toxoplasma tachyzoites were preincubated with increasing concentrations of bombesin for 15 min (A), or PMA for 15 min, or 30 min (B).

The efficacy of invasion was evaluated by microscopic counting of the percentage of infected cells after immunofluorescence staining. Results are expressed as relative percentages, corresponding to the relative increase of cell infection, compared to control values (cell infection by untreated tachyzoites, i.e., 20.2 ± 5.5 % in (A), 18.5 ± 2.1 % in (B)), arbitrarily brought to 100 %. Data are means ± SE of three independent experiments.

by two different methodologies: *i)* by counting infected host cells after overnight culture, which directly reflects the efficacy of invasion, and *ii)* by [^{3}H]-uracil incorporation after 24 hours, which mostly reflects intracellular growth and more undirectly invasion. Although these two techniques do not measure exactly the same event, we present here the results obtained with both methods of quantification, which were comparable for all the drugs tested.

Genistein, a tyrosine kinase inhibitor, had only a moderate inhibitory effect on parasite infectivity, suggesting that tyrosine kinases are not a major signalling pathway involved in cell invasion. Other authors have reported different results with another protozoan parasite, *T. cruzi* (Vieira *et al.*, 1994; Favoreto *et al.*, 1998). They showed that genistein inhibited macrophages infection by *T. cruzi* by up to 90 %. In other studies, genistein prevented proliferation of *T. brucei* (Wheeler-Alm & Shapiro, 1992) and *Listeria* (Velge *et al.*, 1994).

By contrast, tachyzoite stimulation with bombesin, which activates MAP kinases in Swiss-3T3 cells (Pang *et al.*, 1993), had a marked effect on both parasite infectivity and growth, as measured by both techniques of quantification, suggesting that MAP kinases are a major crossing pathway in parasite signalling. In our experiments, tachyzoite activation with bombesin stimulated host cell invasion 2-fold. In addition, preincubation of cell cultures with 50 nM bombesin led to a significant increase in *Toxoplasma* infectivity (not shown), suggesting that signalling pathways involving MAP kinases in both the host cell and the parasite could be involved in the invasion process. Similarly, prestimulation of tachyzoites with PMA, a PKC activator (Kraft & Anderson, 1983), also induced a large increase in infectivity. As PKC is involved in MAP kinase activation (Garrington & Johnson, 1999), these results support a role of MAP kinases in *Toxoplasma* invasion. MAP kinase activation through PMA is a transient and reversible event, which is both time- and dose-dependent. For this reason, the effect of this drug on tachyzoite invasion was observable only when a given concentration was applied for a given time period, i.e. 500 nM for 30 min, as assessed by microscopic counting. Conversely, tachyzoite treatment with RO31-8220, a PKC inhibitor, significantly reduced both parasite infectivity and growth, as shown by the near-abolition of uracil incorporation. Taken together, these results could confirm a role of MAP kinases via PKC activation. It was reported that PMA can increase host cell infection by *T. cruzi* (Vieira *et al.*, 1994), while staurosporine, a PKC inhibitor, has a profound effect on the division and morphology of *Leishmania* promastigotes (Becker & Jaffé, 1997) and inhibits erythrocyte invasion by *P. knowlesi* (Ward *et al.*, 1994), both effects suggesting a role of PKC in kinetoplastid survival and infectivity.

To confirm the increase in infectivity occurring via MAP kinase activation, we preincubated *Toxoplasma* tachyzoites with PD098059, a specific inhibitor of MEK. MEK is an upstream dual-specific MAP kinase kinase that activates the MAP kinases ERK1 and ERK2 by phosphorylation of Thr-183 and Tyr-185 residues (Her *et al.*, 1993). Host cell infection by tachyzoites pretreated with PD098059 was markedly inhibited, pointing to activation of ERK1- or ERK2-like proteins in *T. gondii* invasion process. Similar results have been obtained using another *in vitro* model (Gomez-Marin *et al.*, 1998).

Taken together, our results suggest that ERK-like kinases may be involved in *Toxoplasma* infectivity

and growth, at least *in vitro*, through PKC activation. In other pathogens, such as *Listeria* and *Salmonella typhimurium*, the ERK pathway is required for host cell invasion (Pace *et al.*, 1993; Hobbie *et al.*, 1997; Tang & Sutherland, 1998). The involvement of MAP kinase pathways in the invasion process could be related to various roles of MAP kinases in eukaryotic cells, such as: *i*) cytoskeleton regulation and formation of actin stress fibers through the small GTP-binding protein Rho (Ridley & Hall, 1994), *ii*) activation of PLA2 (Hirasawa *et al.*, 1995; Xing *et al.*, 1996), and *iii*) exocytosis of secretory vesicles (Cox & Parsons, 1997). In particular, the role of MAP kinases in *Toxoplasma* infectivity could be explained in several ways. First, a PLA2 from the parasite is known to be required for host cell penetration (Saffer & Schwartzman, 1991), an enzyme which could be activated by parasite MAP kinases. The invasion process takes place in several stages, including cell-surface recognition, parasite attachment, and exocytosis of specific secretory organelles (rhoptries and micronemes), that could depend on MAP kinase activation. Finally, the parasite actin cytoskeleton is essential for its motility and entry into the host cell, as demonstrated by the inhibition of invasion by cytochalasin D (Dobrowolski & Sibley, 1996). As MAP kinases (also known as microtubule-associated protein kinases) are involved in the polymerization of actin stress fibers through small GTP-binding protein, they may also contribute to parasite motility and invasiveness.

In conclusion, our results point to the involvement of MAP kinase signalling pathways in the host cell invasion by *Toxoplasma gondii*, possibly via PKC activation.

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