Protein kinase C regulation of cell spreading in the molluscan 
Biomphalaria glabrata embryonic (Bge) cell line

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Abstract

Cellular adhesion and spreading are critical components involved in the processes of cell and tissue development, and immune responses in molluscs, but at present, little is known regarding the signaling pathways involved in these basic cellular functions. In the present study, the molluscan Biomphalaria glabrata embryonic (Bge) cell line was used as an in vitro model to study the signal transduction pathways regulating molluscan cell adhesion and spreading behavior. Western blot analysis using antibodies specific to mitogen-activated protein kinase (MAPK) revealed the presence of an MAPK-like immunoreactive protein in Bge cells, that was phosphorylated upon exposure to phorbol myristate acetate (PMA). Moreover, Bge cell treatment with inhibitors of protein kinase C (PKC), Ras and MAPK kinase (Mek) suppressed PMA-induced expression of activated MAPK, suggesting that PKC-, Ras- and Mek-like molecules may be acting upstream of MAPK. Similarly, in vitro Bge cell-spreading assays were performed in conjunction with the same panel of inhibitors to determine the potential involvement of PKC, Ras and Mek in cellular adhesion/spreading. Results revealed a similar pattern of inhibition of cell-spreading behavior strongly implying that the Bge cell spreading also may be regulated through a MAPK-associated signal transduction pathway(s) involving proteins similar to PKC, Ras and Mek. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cell adhesion and spreading are essential for development, maintenance and survival in all multicellular organisms, and are directly involved in such processes as embryogenesis, morphogenesis and tissue organization, and immune responses [1–3]. Although, there exists a substantial body of knowledge regarding the molecular mechanisms underlying the regulation of mammalian cell adhesive behavior, very little is known about the same regulatory mechanisms in molluscs. Previous studies have demonstrated that cells of the molluscan Biomphalaria glabrata embryonic (Bge) cell line could be inhibited in their in vitro spreading behavior by treatment with the tetrapeptide RGDS [4]. RGDS inhibition implied the involvement of integrin adhesion receptors and indeed, in subsequent studies, a \( \beta_1 \)-like integrin subunit cDNA homologue was cloned from Bge cells [4]. Few inver-
tebrate integrin homologues have been discovered to date [5] and therefore our knowledge of integrin-mediated adhesion and signaling mechanisms has stemmed primarily from mammalian cell research. Participation of integrin receptors in cell adhesion and spreading has been well established in numerous mammalian cell types [6–8]. The study of integrin-mediated cellular signaling associated with adhesive behavior likewise has been extensively studied in mammalian systems, and results show that signaling pathways can vary considerably depending on cell type and integrin subunit combination [9]. Despite the potential complexity of signal transduction networks, it has frequently been demonstrated that protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) act downstream of integrin activation, often in a signaling cascade regulating mammalian cell spreading (Fig. 1) [10–13].

Analogous to mammalian systems, recent studies demonstrate a similar role for integrins in invertebrate cell adhesion and migration; for example, the finding by Martin-Bermudo et al. [14] that Drosophila cells migrate via PS integrin receptors. However, integrin-mediated signaling, and signaling pathways in general, in invertebrates remains a comparatively unexplored area. Nevertheless, one may hypothesize that invertebrate signal transduction involves proteins homologous to those found in mammalian systems. In support of this hypothesis, there is a growing accumulation of literature reporting homologous signal transduction molecules in invertebrates, including MAPK, focal adhesion kinase (FAK), Grb2, and Ras homologues in Drosophila and Caenorhabditis elegans [15–18]. In addition, inhibition studies have detected the activity of specific signaling molecules in several molluscs, including arachidonic acid (AA), cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP3), MAPK, protein kinase A (PKA), PKC, and phospholipase A2 (PLA2) [19–23].

The aims of the present study were twofold: first, to examine whether proteins similar to vertebrate signal transduction molecules such as PKC and MAPK might be present in Bge cells, and second, to investigate which signaling molecules and pathways regulate Bge cell spreading. These objectives were carried out using selective inhibitors to a variety of signal transduction molecules, including calphostin C and chelerythrine chloride to block PKC, Ras inhibitory peptide and FTase Inhibitor I to block Ras, and PD 98059 to block Mek.

2. Materials and methods

2.1. Biomphalaria glabrata embryonic (Bge) cell maintenance and isolation

Bge cells, originally isolated by Hansen [24], were obtained from American Type Culture Collection (ATCC CRL 1494). Cells were maintained at 26°C at normal atmospheric conditions in complete Bge cell medium [24], supplemented with 10% heat-inactivated fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO) and antibiotics (100 U/ml penicillin G and 0.05 g/ml streptomycin sulfate). Bge cells, grown to near confluence in 75-cm2 tissue culture flasks, were first rinsed with cold Chernin’s balanced salt solution, pH 7.2 (CBSS) [25], followed by addition of fresh cold (4°C) CBSS and the suspension of attached Bge cells by gentle pipeting of buffer. Suspended cells were centrifuged at 1500 rpm (Centrifuge 5810 R, Eppendorf, Brinkmann Instruments, Westburg, NY) for 3 min at 4°C, resuspended in fresh cold CBSS and counted using a hemocytometer. All chemicals used in this study were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO, unless otherwise indicated.

2.2. Inhibitors

A variety of specific inhibitors were employed in the study: calphostin C and chelerythrine chloride were used to inhibit the regulatory and catalytic domains, respectively, of protein kinase C (PKC). Calphostin C binds to the diacylglycerol (DAG) binding site and selectively inhibits DAG-sensitive PKC isozymes [26], while chelerythrine chloride is a nonspecific inhibitor which competitively inhibits the phosphate acceptor site and noncompetitively inhibits the ATP binding site [27]. Two inhibitors of Ras were utilized; firstly Ras inhibitory peptide (Val-Pro-Pro-Pro-Val-Pro-Arg-Arg-Arg), which hinders Ras activation by preventing the formation of a Grb2-SOS complex [28] and secondly, FTase Inhibitor I (Calbiochem Novabiochem Corp., San Diego, CA), which acts by blocking farnesylation of the Ras pro-
tein thus stopping its translocation to the plasma membrane [29]. Finally, PD 98059 (Calbiochem Novabiochem) was included to inhibit mitogen-activated protein kinase kinase (MAPKK or Mek), by preventing the activation of Mek by Raf, a serine/threonine protein kinase [30].

2.3. MAPK detection and its activation by PMA

Antibodies specific to MAPK and activated (diphosphorylated) MAPK were used in Western blot analyses (see detailed description below) to detect immunoreactive MAPK in Bge cells under conditions of cellular activation and in the presence of signal transduction inhibitory drugs.

In initial PMA-activation experiments, Bge cells were isolated, washed and counted following the protocols described, and 1.5 \times 10^5 cells in 50 \mu l CBSS per well were aliquoted into wells of a 96-well tissue culture plate (Costar, Corning, NY). Cells were allowed to spread for 3 h, followed by centrifugation at 1000 rpm for 1 min at room temperature and removal of supernatant (Centrifuge 5810 R, Eppendorf, Brinkmann Instruments). Following an additional 30-min incubation in CBSS, cells were treated with either 50 \mu l 500 nM phorbol 12-myristate 13-acetate (PMA) or 50 \mu l CBSS (negative control). After 10 min incubation, the plate was centrifuged as before, the supernatant removed, and cells were lysed with 2\times sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) reducing buffer diluted 1:1 with CBSS. Samples were boiled for 5 min and stored at \(-20^\circ C\) until Western blot analysis.

2.4. Inhibition of PMA-mediated MAPK activation

In order to test the hypothesis that MAPK activation is regulated upstream by PKC or Ras signal transduction pathways, various drugs targeting specific signal pathway components were used to block PMA-mediated activation of Bge cell MAPK. A similar protocol as outlined above was used. Briefly, 1.5 \times 10^5 cells were aliquoted into wells of a 96-well tissue culture plate and allowed to spread for 3 h. The plate was then centrifuged, the supernatant removed and the cells were treated with either 50 \mu l inhibitor or 50 \mu l CBSS (negative control) for 30 min. Following treatment cells were stimulated with either 50\mu l 500 nM PMA or 50 \mu l CBSS for 10 min. Plates were then centrifuged as before, the supernatants removed, and cells were lysed with 2\times SDS-PAGE reducing buffer diluted 1:1 with CBSS. Samples were boiled for 5 min and stored at \(-20^\circ C\) until Western blot analysis. One exception to the protocol described above regards the use of FTase Inhibitor I, whereby cells were aliquoted and allowed to spread in the presence of inhibitor for 3 h, prior to stimulation with PMA. This 3 h pre-PMA incubation period was carried out in order to deplete levels of previously farnesylated, membrane bound Ras protein.

Viability of control and drug treated cells was tested using the trypan blue exclusion method, in which cells were aliquoted and allowed to spread for 3 h, followed by replacement of supernatant with either 50 \mu l inhibitor or CBSS control. After 30 min, trypan blue was added to the wells and the percentage viable (unstained) cells calculated.

2.5. Western blot analysis

After solubilization of cells in SDS–PAGE reducing buffer, samples were loaded onto a 10% polyacrylamide gel, separated at 150 mV for 45 min, and subsequently transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK) at 100 mA for 30 min. Membranes were incubated overnight at 4^\circ C in a blocking solution of TBS (10 mM Tris–HCl, 100 mM NaCl, 0.2% Tween 20, pH 7.5) containing 0.1% bovine serum albumin. To detect MAPK (both activated and nonactivated forms), membranes were incubated with a polyclonal anti-MAPK (Erk 1/2, Sigma–Aldrich) antibody at a dilution of 1:20 000 for 2 h. Membranes were then washed 2\times 15 min in TBS, and incubated in a horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Promega Corp., Madison, WI) at a dilution of 1:20000 for 2 h. Membranes were then washed 2\times 15 min in TBS, and incubated in a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Promega Corp., Madison, WI) at a dilution of 1:25000 for 1 h. To detect activated (diphosphorylated) MAPK, membranes were incubated with an anti-diphosphorylated MAPK (Erk 1/2, Sigma–Aldrich) monoclonal primary antibody for 2 h at a dilution of 1:100000. The latter antibody specifically recognizes activated or diphosphorylated (Thr183 and Tyr185) MAPK (Erk
After 2 × 15-min TBS washes, the blots were incubated in an HRP-conjugated rabbit anti-mouse IgG (H+L) secondary antibody (Pierce, Rockford, IL) at a dilution of 1:20000 for 1 h. Membranes were again washed 2 × 15 min in TBS. Subsequently, all blots were incubated 5 min in 10 ml SuperSignal West Pico Chemiluminescent Substrate (Pierce), followed by exposure to radiographic film for approximately 30 sec. The film was developed in Kodak GBX developer, washed in double-distilled water, then fixed in Kodak GBX fixer (Eastman Kodak Co., NY). A nerve growth factor (NGF)-treated PC12 cell extract (Promega) was included as a positive control, as 42/44 kDa MAPK has been shown...
to be activated (diphosphorylated) following NGF treatment [31]. Each experiment was repeated four times.

2.6. Cell-spreading assays

Approximately $1 \times 10^4$ Bge cells, isolated and enumerated as described previously, were aliquoted in triplicate to wells of 24-well Teflon-printed glass microslides (Cel-line Associates, Newfield, NJ). Cells were allowed to attach and spread 30 min in the presence of either; an inhibitor over a range of concentrations or CBSS saline control. The inhibitors used, and the range of concentrations tested, were as follows: calphostin C (0.5–20 nM), chelerythrine chloride (5–100 nM), Ras inhibitory peptide (1–100 μM), FTase Inhibitor I (1–100 μM) and PD 98059 (100–750 μM). Cells were then fixed in 4% paraformaldehyde (PFA) for 5 min, mounted in CBSS, and examined at 400× using an Olympus BH-2 phase-contrast microscope. A minimum of 200 cells per well were counted and the percentage of spread cells was calculated. Four independent replicates of each experiment were performed. Data were then analyzed by one-way analysis of variance followed by Dunnett’s post hoc test using the software package Graph Pad Prism version 3.0. Viability of control and treated cells was assessed via the trypan blue exclusion method as previously described.

3. Results

3.1. MAPK assay: detection of MAPK and its activation in Bge cells

Using the general anti-MAPK polyclonal antibody, a single protein band of approximately 42 kDa molecular mass was detected, in both the control samples incubated in CBSS alone and in cell samples stimulated with 250 nM PMA (Fig. 2a). The monoclonal anti-MAPK antibody, which specifically recognizes activated or diphosphorylated MAPK, cross-reacted with a single protein band of the same molecular mass only in samples of PMA-stimulated Bge cells and not in the control lanes representing cells treated with CBSS alone (Fig. 2b). Both antibodies also recognized the activated MAPK standard, which comigrated with the Bge cell MAPK-like protein band. The two protein bands visible in Fig. 2b (lane 1) represent ERK1 and ERK2 (44 and 42 kDa) of the MAPK family.

In order to investigate the possible cell signaling pathways responsible for PMA-mediated MAPK activation, cells were stimulated with PMA after pre-incubation with inhibitors to various signal transduction molecules. All the inhibitors employed were capable of reducing PMA-stimulated MAPK-like protein phosphorylation at different concentrations. Calphostin C was inhibitory at concentrations of 1 nM and above (Fig. 3a), whilst concentrations equal to or greater than 10 nM chelerythrine chloride significantly inhibited the activation of MAPK (Fig. 3b). Incubation of Bge cells with 50 μM or greater Ras inhibitory peptide also significantly inhibited MAPK phosphorylation, while it took FTase Inhibitor I twice the concentration to bring about the
same level of MAPK inhibition (Fig. 4a,b). PD 98059, which blocks Mek, also inhibited MAPK phosphorylation, but only at comparatively high concentrations of 50 μM or greater (Fig. 5). Viability of drug-treated cells did not differ significantly from cells under control conditions, confirming that the inhibition of MAPK phosphorylation was induced by the different drugs, and not an artifact of cell death.

3.2. Bge cell-spreading assays

All inhibitors employed in the study significantly reduced Bge cell spreading although in varying degrees depending on inhibitor concentrations. On comparison, the inhibitor concentrations required to elicit an effect on Bge cells was found to correspond with those used in mammalian systems [28–30,32,33]. Bge cells responded in a typical dose-dependent manner to a majority of the inhibitors with the exception of the Ras inhibitory peptide, which did not exert a significant effect on spreading until the highest concentration was reached, at which point cell spreading was almost completely inhibited. The most potent inhibitor was found to be the PKC inhibitor, calphostin C, for which significant inhibition of spreading was seen at concentrations as low as 0.5 nM (P ≤ 0.05) (Fig. 6a). Chelerythrine chloride,
the other PKC inhibitor, exerted a significant inhibitory effect on cell spreading with a lowest significant inhibitory concentration (LSIC) of 25 nM \((P \leq 0.01)\) (Fig. 6b). As shown in Fig. 7a,b, Bge cells responded to both Ras inhibitors but with different behavioral patterns. Spreading was gradually inhibited in the presence of FTase Inhibitor I starting at 1 \(\mu\)M with maximum spreading inhibition attained by 50 \(\mu\)M. In contrast, no significant inhibitory effect was seen with the Ras inhibitory peptide from concentrations from 1 to 50 \(\mu\)M. However at 100 \(\mu\)M Bge cell spreading was reduced from 84.4% in the CBSS control to 7.9% in peptide-treated cells (Fig. 7a). Finally, similar to the Ras inhibitory peptide, the minimum inhibitory dose for PD 98059 was 100 \(\mu\)M (Fig. 8), although the effect was much less than Ras inhibitory peptide at the same concentration. Bge cells exposed to 100 \(\mu\)M Ras inhibitory peptide exhibited 7.9% spreading compared to 51.2% with 100 \(\mu\)M PD 98059. It is important to note that results of Bge cell viability assays demonstrated that the reduction in cell spreading was not due to drug-induced cytotoxic effects of the inhibitors employed in these experiments.

4. Discussion

Very little is known about specific signaling molecules and pathways in cells of the gastropod \emph{B. glabrata}, although a number of recent studies are amongst the first to bridge this void. Hertel et al.
observed cell rounding and intracellular calcium transients when *B. glabrata* hemocytes (circulating phagocytic cells) were exposed to larval *Echinostoma paraensei* excretory-secretory products, while treatment with PMA induced calcium transients without rounding. This implies that *Biomphalaria* cells are capable of signaling through calcium fluxes, and modulating the cytoskeletal architecture of the cell in response to chemical stimuli. In addition, a receptor for activated protein kinase C (RACK) has also been identified in *B. glabrata*, and detected immunocytochemically in both hemocytes and Bge cells [35]. Therefore, the present study focused on identifying potential signaling molecules in the molluscan Bge cell line, and secondly, to determine whether the MAPK signal transduction pathway(s) may be involved in regulating in vitro cell spreading.

As a β1-like integrin subunit has been identified in Bge cells [4], it was subsequently hypothesized that Bge cells might possess some of the signaling molecules known to act downstream of integrin activation in mammalian cells. Mitogen-activated protein kinase (MAPK), of the serine/threonine protein kinase family, is frequently cited as a primary participant in integrin receptor mediated signal transduction [36–38] and, therefore, it was hypothesized that a MAPK-like protein may be present in Bge cells, as well as a protein kinase C (PKC) intracellular signaling system involved in regulating MAPK activation. Results of Western blot analysis employing specific MAPK antibodies revealed the presence of an immunoreactive MAPK-like molecule with a molecular mass of 40–44 kDa, in close proximity to the 44 and 42 kDa ERKs 1 and 2 [39]. Moreover, as PD 98059 is known to inhibit MAPKK or Mek, which directly catalyzes MAPK diphosphorylation, inhibition of Bge cell MAPK activation by PD 98059 further supports the presence of a MAPK-like protein in Bge cells.

In order to better understand the function of *Biomphalaria* MAPK, the signaling molecules and pathways that might be acting upstream of MAPK, regulating its activation, were investigated. Western blot analyses showed that MAPK could be activated/phosphorylated following PMA treatment, which is evident of a PKC signaling pathway since PMA is a diacylglycerol (DAG) analogue that is known to activate DAG-sensitive PKC isozymes [40]. Furthermore, MAPK phosphorylation was inhibited upon incubation with very low concentrations of specific PKC inhibitors such as calphostin C and chelerythrine chloride; for example inhibitory concentrations for calphostin C (0.5 nM) were approximately 400 times less than required to block human neutrophil adhesion [33]. Calphostin C acts by interfering with DAG binding to the cysteine-rich (C1) regulatory domain of PKC [26], while chelerythrine chloride targets the PKC catalytic site, but is not DAG-specific [27]. Thus calphostin C inhibition of PMA-mediated activation of MAPK in Bge cells suggests that the PKC-like protein in Bge cells is probably closely related structurally to a DAG-sensitive PKC isozyme(s). Upon discovering that MAPK could be activated by PMA, presumably through a PKC pathway, additional inhibitors were used to investigate which signal transduction molecules might also be involved. PMA-induced MAPK phosphorylation was reduced following treatment with Ras inhibitory peptide, FTase Inhibitor I and PD 98059, implying Ras and Mek may also act upstream of MAPK in this PKC/MAPK signaling cascade.

To further characterize the functional role of MAPK, and specifically its involvement in the regulation of cellular spreading, in vitro cell-spread assays in conjunction with the same panel of specific inhibitors of signal transduction molecules were performed. The cumulative inhibition profiles demonstrated that proteins similar to PKC, Ras and Mek
also regulate spreading in Bge cells. As Mek acts directly upstream of MAPK in the PKC/MAPK signaling cascade, inhibition of spreading by PD 98059 (Mek inhibitor) also implicates MAPK involvement in cell-spreading behavior. Our findings are consistent with mammalian cell studies in which PKC and Mek activation of MAPK were responsible for mediating cell spreading in a number of cell lines. For example, the spreading behavior of a colon carcinoma cell line was enhanced upon PMA treatment, and displayed sensitivity to PKC inhibitors [11] while the Mek inhibitor UO126 hindered spreading of rat fibroblasts [41].

As mentioned previously, recent studies have suggested that Bge cell spreading is regulated through an integrin-like receptor(s) [4]. This finding is supported by a growing body of literature that indicates similar integrin receptor involvement in cell adhesive and spreading behavior in a variety of mammalian cell types [6,8,42]. Furthermore, both PKC and MAPK are also reported to act downstream of integrin-mediated signaling, as observed in breast cancer and fibrosarcoma cells, where plasminogen activation of MAPK occurs through a β1 integrin, activating Ras and Mek [43]. PKC/MAPK regulation of integrin-mediated signal transduction parallels that observed in Bge cells, where the current data strongly implies regulation of cell-spreading behavior through a PKC/MAPK signaling pathway. A hypothetical pathway delineating regulation of Bge cell spreading is presented here whereby, spreading is initiated by integrin engagement and clustering, followed by activation of PKC by DAG (Fig. 1). FAK is activated by PKC and, after autophosphorylation, is able to bind Grb2 by its SH2 domain. Importantly, inhibition of MAPK activation and Bge cell spreading by the Ras inhibitory peptide suggests the presence of a molluscan Grb2/SOS-like mechanism. According to this mechanism, an adapter protein, Grb2 carries SOS to Ras, which is activated by the exchange of GTP for GDP [44]. Ras may then activate Raf, which in turn phosphorylates Mek, which phosphorylates MAPK on Thr183 and Tyr185. Induction of cell spreading is presumed to be mediated by MAPK activation of phospholipase A2 (PLA2), resulting in an increase in arachidonic acid (AA) levels [45].

The inhibitors employed in the present study have been used in numerous similar studies [30,32–34] and, consequently have been subjected to thorough evaluation of inhibitory mechanisms. However, caution has been applied when interpreting the present inhibitor studies since the observation of an inhibitory effect does not prove that the drug is acting by the same mechanism as in other diverse systems. To add needed evidence in support of our Bge cell inhibitor experiments, future studies will include the identification and characterization of Biomphalaria signal transduction molecules at the molecular level. For example, recently, we have cloned a partial cDNA sequence encoding a MAPK-like protein in Bge cells (J.E. Humphries, T.P. Yoshino, unpublished data).

In conclusion, the results of this study suggest that molluscan B. glabrata embryonic cells possess an MAPK-like protein and that PKC- and/or Ras-like signal transduction molecules may regulate its activation. Secondly, these same signaling molecules and their pathways may be responsible for regulating in vitro Bge cell spreading. Bge cell spreading probably is not governed by a single, linear pathway but more likely is under the regulation of an intricate signaling network, in which multiple signaling pathways would interact with each other, transferring signals received from various receptor–ligand interactions. Further detailed studies are required to elucidate the exact mechanisms regulating Bge cell spreading, ultimately providing an insight into cell and tissue development and organization in molluscs. This is the first demonstration of PKC, Ras and MAPK-like signaling molecules in the snail, B. glabrata. It is of great interest to explore the level of homology and conservation between B. glabrata signaling molecules and those already characterized from vertebrates, at both the structural and functional levels.

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