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Involvement of the muscarinic acetylcholine receptor in inhibition of cell migration

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Abstract

Activation of G protein-coupled receptors is known to stimulate cell migration, but receptor-mediated signals inhibiting cell migration have not been identified. We investigated the ability of transfected human M_3 muscarinic acetylcholine receptors (mAChR) to regulate the migration of Chinese hamster ovary (CHO) cells. Single cells migrated on colloidal gold applied to fibronectin-coated plates, and videomicroscopy was used to measure cell spreading and migration. Activation of M_3 mAChR with the agonist carbachol was found to inhibit cell migration, whereas direct activation of protein kinase C (PKC) with PMA was found to stimulate migration. The amount of cell adhesion and spreading was found to be equivalent for carbachol- and PMA-treated cells. Selective inactivation of conventional PKC isoforms with Go6976 ($C_{24}H_{18}N_4O$) abolished the PMA-mediated increase in cell migration. In contrast, the mAChR-mediated decrease in migration was not altered by Go6976, but was abolished when both novel and conventional PKC isoforms were inactivated by calphostin C or chelerythrine. These findings suggest involvement of conventional PKC isoforms in the stimulation of migration and of novel PKC isoforms in the inhibition of migration. Carbachol- but not PMA-treated cells exhibited an elongated morphology reminiscent of migrating cells that cannot detach their trailing edges from the substratum. Similarly, carbachol-treated cells detached less readily from fibronectin than control or PMA-treated cells when integrin activity was diminished by the chelation of Ca^{2+} and Mg^{2+}. Finally, the carbachol-induced diminution of cell detachment was preserved after inhibition of the conventional PKC isoforms with Go6976, but was abrogated by treatment with either calphostin C or chelerythrine. These findings suggest that mAChR activation diminishes the ability of cells to detach from the substratum, resulting in diminished migration. This is in contrast to the direct activation of PKC with PMA, which stimulates migration.

Keywords: Calphostin C; Cell adhesion; Cell detachment; Cell migration; Chelerythrine; Go6976; Muscarinic acetylcholine receptor; Protein kinase C isoforms

1. Introduction

Cell migration is involved in many normal and pathological processes, including immune function (reviewed in [1]), wound healing (reviewed in [2]), repair of endothelial damage in blood vessels [3], and cancer metastasis (reviewed in [1,4]). Elucidating the signals that regulate cell migration would potentially allow the development of specific therapies to control these processes. Cell migration can be increased by activating a variety of heterotrimeric G protein-coupled receptors, including those for lysophosphatidic acid [5–7], sphingosine 1-phosphate [6–9], opioids [10,11], thrombin [5,7,10], angiotensin II (reviewed in [12]), adenosine [13], and multiple peptide chemoattractants and cytokines (reviewed in [1,10]). Some of these receptors stimulate $G_{q/11}$ proteins, whereas others stimulate G_i proteins (reviewed in [5,10–12]). Thus, multiple signaling pathways induce cell migration.

In contrast to signals that stimulate migration, surprisingly little is known about signaling pathways that inhibit cell migration. Heterotrimeric G protein-coupled receptors that can inhibit cell migration have not been identified previously. The cessation of migration under certain conditions, such as when cells contact one another [14], indicates that cells possess signaling pathways to diminish migration. However, the receptor-mediated signals that diminish cell migration have not been characterized.

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Abbreviations: CHO, Chinese hamster ovary; ECM, extracellular matrix; mAChR, muscarinic acetylcholine receptors; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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The migratory rate of cells depends upon the activity of integrin adhesion molecules (reviewed in [2,15]). Integrin activation may stimulate migration because the interaction of integrins with the extracellular matrix (ECM) provide cell traction. However, too high integrin activity may prohibit migration because cells cannot release from the ECM and move forward (reviewed in [2,15]). These findings suggest that cells will not migrate when they have exceedingly low or high integrin activity. Thus, signals that induce these extreme states of integrin activity may diminish migration.

PKC is believed to regulate cell migration by regulating the activity of integrins [16,17]. Activation of PKC with phorbol 12-myristate 13-acetate (PMA) enhances $\alpha_5\beta_1$ integrin activity in CHO cells, and stimulates the migration of these cells [16]. Activation of PKC also stimulates the migration of many other cell types, including polymorphonuclear cells, monocytes, endothelial cells (reviewed in [3]), and retinal pigment epithelial cells [18]. These findings indicate that receptors that regulate PKC are potential candidates to regulate cell migration.

Activation of M_1 or M_3 mAChR stimulates PKC activity (reviewed in [19–21]) and induces integrin-mediated adhesion [22]. These receptors are members of the mAChR family that includes five receptor subtypes. The odd-numbered mAChR subtypes (M_1, M_3, and M_5) activate PKC by stimulating $G_{q/11}$ proteins. In contrast, the even-numbered subtypes (M_2 and M_4) diminish protein kinase A (PKA) activity by stimulating G_i (reviewed in [19–21]). The ability of M_1 and M_3 mAChR to regulate PKC and integrin activity prompted us to characterize the ability of these receptors to regulate CHO cell migration.

Our results indicate that activation of transfected M_1 or M_3 mAChR inhibits CHO cell migration. Single cells that are not in contact with other cells exhibit this response, indicating that the mAChR-mediated decrease in cell migration does not depend upon changes in cell–cell adhesion. We investigated the role of different PKC isoforms in the M_3 mAChR-mediated inhibition of migration using selective PKC antagonists. Inactivation of conventional PKC isoforms with the selective antagonist Go6976 ($C_{24}H_{18}N_4O$) [23] does not abolish the mAChR-mediated inhibition of migration, suggesting that conventional PKC isoforms are not required for this process. However, inactivation of novel as well as conventional PKC isoforms with calphostin C [24] does abolish the ability of the M_3 mAChR to inhibit migration. These results suggest that novel PKC isoforms may participate in the inhibition of migration mediated by M_3 mAChR.

Previous studies demonstrated that the release of integrin-ECM bonds at the rear of the cell determines the rate of migration [25,26]. Cells with impaired rear release exhibit enhanced ECM adhesion and an elongated morphology [25,26]. We found that activation of transfected M_3 mAChR in CHO cells is also associated with this enhanced adhesion and an elongated phenotype. These data suggest that mAChR activation creates impaired rear release from the ECM, thus inhibiting cell migration.

2. Materials and methods

2.1. Cell lines and reagents

CHO-K1 sublines stably transfected with the M_1, M_2, or M_3 subtype of human mAChR are referred to as CHO-m1, CHO-m2, or CHO-m3, respectively. These sublines have been described previously [21]. All cells were maintained in complete CHO medium consisting of Ham's F-12 medium (Mediatech), heat-inactivated fetal bovine serum (5%) (BioWhittaker), glutamine (0.3 μg/mL), penicillin (20 units/mL), and streptomycin sulfate (20 μg/mL). Cells were maintained at 37° in a humidified atmosphere of 5% CO_2, 95% air. Fibronectin, carbachol, and PMA were purchased from the Sigma–Aldrich, and [^{3}H]thymidine (60 Ci/mmol) was obtained from ICN. The kinase inhibitors calphostin C, chelerythrine, and Go6976 were obtained from Calbiochem. Microtiter plates came from the Costar Corp. Other reagents were obtained from sources listed in the text.

2.2. Cell migration and spreading

Cell migration and spreading were measured using a phagokinesis assay [27]. Microtiter plates were coated with different concentrations of fibronectin dissolved in PBS by placing 50 μL of the fibronectin solution in each well. After overnight incubation at 25°, each well was washed once with 200 μL PBS and allowed to air dry. A solution of colloidal gold was prepared as previously described [27] and added to the wells of the fibronectin-coated plate. The wells were incubated for 30 min at 25° and washed with Ham's medium. Two hundred cells in 100 μL of complete CHO medium were added to each well, and incubated for 30 min at 37°. The PKC antagonists (dissolved in complete CHO medium) were added to the wells, and the cells were incubated for an additional 30 min. The cells were then incubated with 10 μM carbachol (in complete CHO medium), 10 nM PMA (in complete CHO medium), or no drug (complete CHO medium alone) for 4 hr. After fixation in paraformaldehyde (3% in PBS), the cells were incubated with crystal violet (0.5% in 50% methanol) for 30 min at 25°, and washed three times with paraformaldehyde (3% in PBS). Ten cells in each of two wells were examined by videomicroscopy using the NIH Image Software program. The outline of each cell was used as an indication of cell spreading, whereas the area of gold particles cleared by each cell was used as an indication of cell migration [27]. Cells that were in contact with other cells, or whose migration trails crossed those of other cells, were not included in the analysis.

2.3. Measurement of cell adhesion

Cells were labeled with [^{3}H]thymidine for 24 hr, as previously described [22], and washed three times in Ham's F-12 medium containing 1% bovine serum albumin (Sigma–Aldrich). The cells were then added to microtiter plates that had been coated with 0.1 μg/mL of fibronectin (2×10^4 cells per 100 μL of culture medium per well), and incubated in the absence or presence of drugs for 40 min at 37°. Non-adherent cells were removed by shaking the inverted plates. Then the wells were washed twice with PBS. The remaining adherent cells were incubated with trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA) for 20 min to lift them from the plates, and collected on filters using the Skatron cell harvester (Skatron Instruments Inc.). The filters were placed in Ultima-Gold scintillation fluid (Packard), and radioactivity was assessed with a Beckman LS-7000 β-counter.

2.4. Measurement of cell detachment

Cells were labeled with [^{3}H]thymidine for 24 hr, as previously described [22], and washed three times in complete medium. The cells were incubated in fibronectin-coated microtiter plates in the absence or presence of PKC inhibitors for 30 min at 37°, followed by incubation in the presence or absence of carbachol or PMA for 3 hr at 37°. The medium was removed from the wells and replaced with 150 μL PBS containing 5 mM EDTA and 5 mM EGTA. The plates were incubated for 3 min at 37° to induce detachment of the cells from the fibronectin-coated plates. Detached cells were collected on filters, and their radioactivity was measured as described above.

2.5. Statistical analyses

The means of the measured values of each treatment group were compared by Student's *t*-test. Means were considered to be significantly different from one another if $P < 0.05$ in Student's *t*-test. In all cases, original measured values, rather than percentages, were used in the analyses.

3. Results

3.1. Differential regulation of cell migration by mAChR and PKC isoforms

We tested the ability of transfected mAChR subtypes to regulate the migration of CHO cells. Incubation for 4 hr with carbachol, an agonist for all mAChR subtypes, inhibited migration of the CHO-m1 and CHO-m3 cell lines (Fig. 1). However, the migration of CHO-m2 cells and untransfected CHO cells was not inhibited by carbachol treatment (Fig. 1). Our finding that cell migration was inhibited only by mAChR subtypes that activate PKC suggests that PKC activation inhibits cell migration. However, we found that direct activation of PKC with PMA stimulated migration in all the cell lines tested (Fig. 1).

To investigate the role of PKC in cell migration further, we studied the effects of PKC antagonists on the motility of CHO-m3 cells. Calphostin C is an antagonist of both conventional (α, β, γ) and novel (δ, ϵ, η, θ) PKC isoforms [24]. Chelerythrine similarly antagonizes conventional and novel PKC isoforms [28]. In contrast, Go6976 inhibits conventional but not novel PKC isoforms [23]. Although

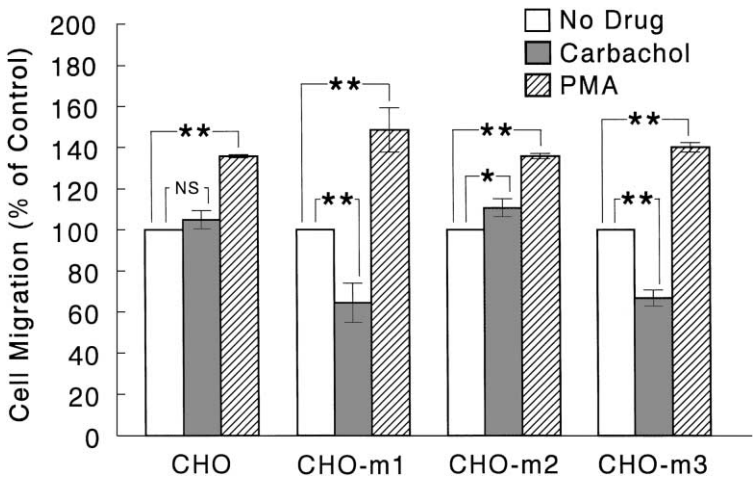

Fig. 1. Inhibition of the migration of CHO-m1 and CHO-m3 cells by treatment with carbachol. Untransfected CHO cells and CHO-m1, CHO-m2, and CHO-m3 cells migrated for 4 hr over colloidal gold on 0.1 μg/mL of fibronectin in the absence or presence of 10 μM carbachol or 10 nM PMA. Control cells migrated in the absence of any drugs. The cells were examined by videomicroscopy. The area cleared of colloidal gold by each cell was used as an indication of cell migration. Results represent the means (±SEM) of the distance migrated by 60 or more cells observed in three independent experiments (20 or more cells observed per experiment). Brackets between bars indicate statistical comparison of the means. Brackets containing asterisks indicate significant differences ($^*P < 0.05$; $^{**}P < 0.01$). NS indicates that the difference between means was not significant ($P > 0.05$).

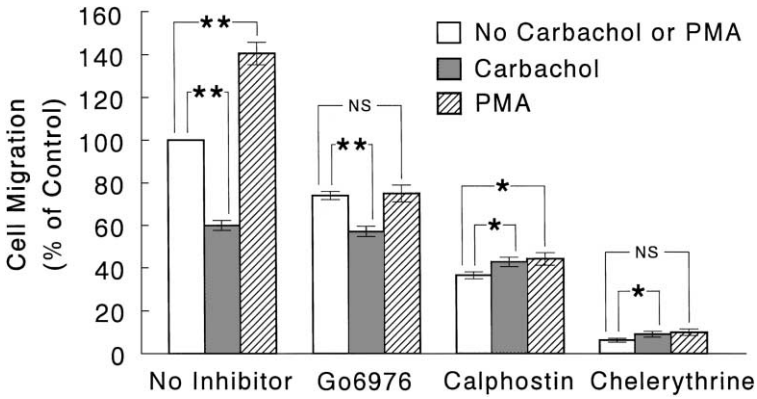

Fig. 2. Effects of PKC inhibitors on cell migration in the absence or presence of carbachol or PMA. CHO-m3 cells were incubated in the absence or presence of 2 μM Go6976, 4 μM calphostin C, or 10 μM chelerythrine for 30 min, and then allowed to migrate in the absence or presence of 10 μM carbachol or 10 nM PMA for 4 hr. Control cells were incubated in the absence of any drugs. Migration was measured as described in the legend of Fig. 1. Results represent the means (±SEM) of the distance migrated by 120–160 cells observed in six to eight independent experiments (20 cells observed per experiment). Brackets between bars indicate statistical comparison of the means. Brackets containing asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$). NS indicates that the difference between means was not significant ($P > 0.05$).

highly selective for certain PKC isoforms, these antagonists will inactivate other kinases if they are used at extremely high concentrations. To avoid this problem, we previously determined the lowest concentrations of the PKC antagonists that abolish M_3 mAChR-mediated myosin reorganization in the CHO-m3 cells: 2 μM Go6976, 4 μM calphostin C, and 10 μM chelerythrine [21]. These concentrations are significantly lower than those needed to inactivate other kinases, such as PKA or myosin light chain kinase (reviewed in [21]). The use of these low but effective antagonist concentrations diminishes the possibility that non-specific inactivation of kinases is occurring.

We found that chelerythrine dramatically inhibited the ability of the CHO-m3 cells to migrate (Fig. 2). Selective inactivation of conventional PKC isoforms with Go6976 significantly diminished basal migration and abolished PMA-stimulated migration. Similarly, inactivation of conventional and novel PKC isoforms with calphostin C significantly diminished basal and PMA-stimulated migration of these cells (Fig. 2). These findings suggest that conventional PKC isoforms are required for both basal and PMA-stimulated migration.

Interestingly, selective inactivation of conventional PKC isoforms with Go6976 diminished but did not abolish the ability of carbachol to inhibit migration (Fig. 2), indicating that the mAChR-mediated inhibition of migration can occur when conventional PKC isoforms are inactivated. This finding suggests that conventional PKC isoforms may not be required for the mAChR-mediated inhibition of migration. In contrast to the effects of Go6976, inactivation of both conventional and novel PKC isoforms with calphostin C abolished the ability of carbachol to inhibit migration (Fig. 2), suggesting that novel PKC isoforms participate in the inhibition of migration by carbachol.

3.2. Effect of fibronectin concentration on cell migration

To investigate the effects of fibronectin concentration on the carbachol- and PMA-mediated changes in cell migration, we measured the migration of cells exposed to different concentrations of fibronectin (Fig. 3). Untreated CHO-m3 cells migrated most quickly on fibronectin concentrations between 1 μg/mL ($P < 0.05$, $N = 60$) and 5 μg/mL ($P < 0.05$, $N = 60$) (Fig. 3). The stimulatory effects of PMA on migration were most apparent when cells were exposed to fibronectin concentrations between 0.5 μg/mL ($P < 0.05$, $N = 60$) and 1 μg/mL ($P < 0.05$, $N = 60$). Interestingly, PMA-treated cells no longer exhibited significantly increased migration when they were plated on fibronectin concentrations of 5 μg/mL or greater

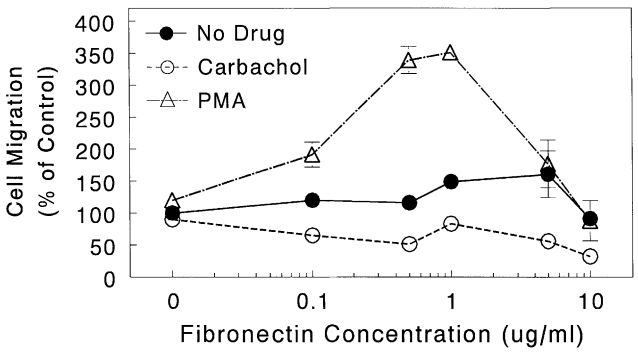

Fig. 3. Effects of fibronectin concentrations on cell migration in the absence or presence of carbachol or PMA. Microtiter plates were coated with the indicated fibronectin concentrations. Migration of CHO-m3 cells for 4 hr in the absence or presence of 10 μM carbachol or 10 nM PMA was ascertained as described in the legend of Fig. 1. Control cells were plated in the absence of fibronectin and incubated in the absence of any drugs. Results are the means (±SEM) of the distance migrated by 60 cells observed in three independent experiments (20 cells observed per experiment).

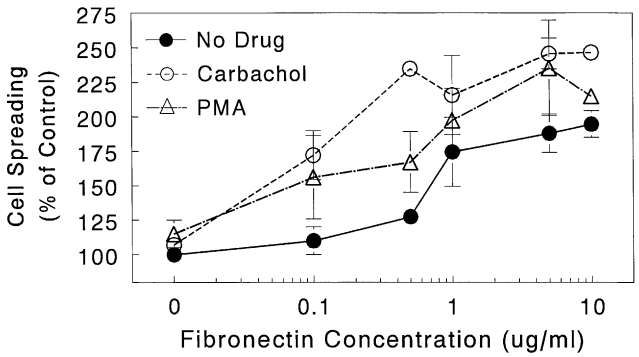

Fig. 4. Effects of carbachol and PMA on cell spreading. CHO-m3 cells were allowed to migrate in the absence or presence of 10 μM carbachol or 10 nM PMA for 4 hr, as described in the legend of Fig. 1, and examined using videomicroscopy. The outline of each cell was used as an indication of cell spreading. Control cells were incubated in the absence of carbachol or PMA, at a fibronectin concentration of zero. Data represent the means (±SEM) of the sizes of 60 cells observed in three independent experiments (20 cells per experiment).

(Fig. 3). Carbachol treatment diminished migration significantly regardless of the fibronectin concentration ($P < 0.05$, $N = 60$) (Fig. 3).

3.3. Induction of cell spreading and adhesion by carbachol and PMA

Cell migration depends upon the ability of cells to adhere to the ECM and spread. Thus, we investigated the effects of mAChR and PKC activation on these processes. Because of the opposite effects of carbachol and PMA on migration, we anticipated that they would also have opposite effects on spreading and adhesion. However, we found that both carbachol and PMA stimulated CHO-m3 spreading ($P < 0.05$, $N = 60$ for all fibronectin concentrations tested except for 1 μg/mL) (Fig. 4). Both drugs also stimulated CHO-m3 adhesion ($P < 0.05$, $N = 16$ for all drug concentrations tested) (Fig. 5).

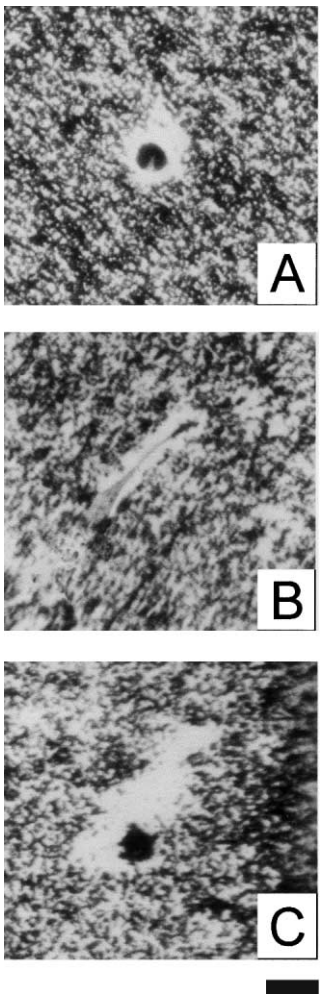

Fig. 6. Elongated morphology of carbachol-treated cells after migration on fibronectin. CHO-m3 cells were plated on 0.1 μg/mL of fibronectin coated with colloidal gold, and allowed to migrate for 4 hr in the absence (A) or presence of 10 μM carbachol (B) or 10 nM PMA (C). The cells were stained with crystal violet and photographed. Representative results are shown.

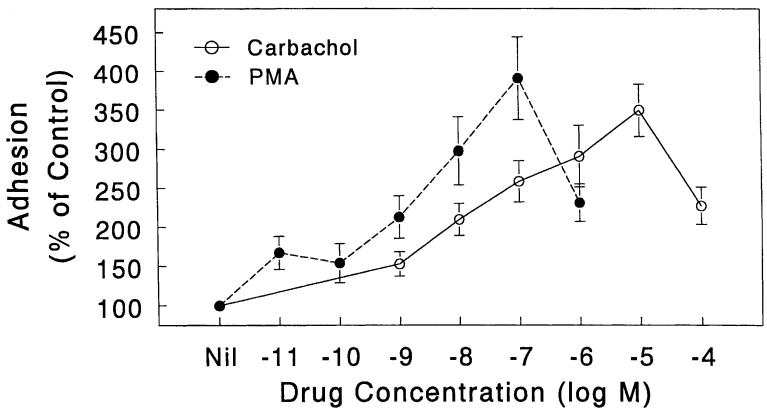

Fig. 5. Effects of carbachol and PMA on cell adhesion. CHO-m3 cells labeled with [^{3}H]thymidine were plated on 0.1 μg/mL of fibronectin and incubated for 40 min in the presence or absence of graduated concentrations of carbachol or PMA. Control cells were incubated in the absence of carbachol or PMA. After removal of non-adherent cells by shaking the plates, the remaining adherent cells were collected and quantitated by β-scintillation counting. Values represent the means (±SEM) calculated from four independent experiments in which each control and experimental group was tested in quadruplicate samples of 2×10^4 cells per sample.

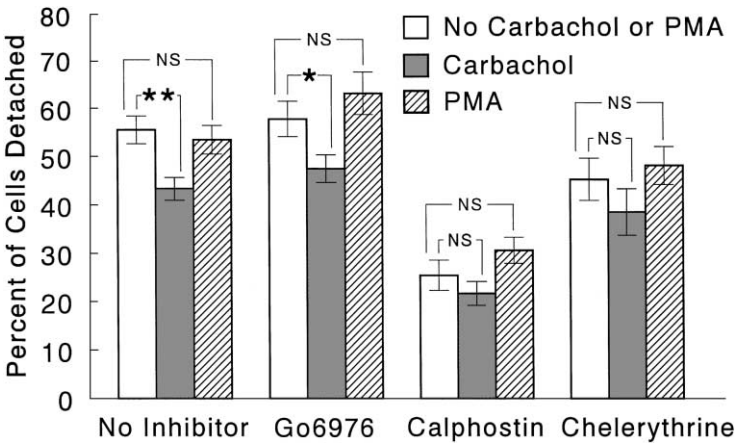

Fig. 7. Effects of PKC inhibitors on cell detachment in the absence or presence of carbachol or PMA. CHO-m3 cells were incubated in the absence or presence of 2 μM Go6976, 4 μM calphostin C, or 10 μM chelerythrine (each in complete CHO medium) for 30 min. Cells were then incubated in the absence or presence of 10 μM carbachol or 10 nM PMA (in complete CHO medium) for 3 hr. Incubated cells were exposed to EGTA/EDTA for 3 min, and detached cells were collected and quantitated by β-scintillation counting. Values are expressed as the percent of total cells plated that subsequently detached. Values represent the means (±SEM) calculated from five independent experiments in which each control and experimental group was tested in quadruplicate samples of 2×10^4 cells per sample. Brackets between bars indicate statistical comparison of the means. Brackets containing asterisks indicate significant differences ($^*P < 0.05$; $^{**}P < 0.01$). NS indicates that the difference between means was not significant ($P > 0.05$).

3.4. Differential regulation of cell detachment by carbachol and PMA

It is known that a migrating cell adopts an elongated morphology because the leading edge of the cell spreads forward while the rear of the cell remains attached to the substratum. Migration occurs when the rear of the cell detaches from the substratum and retracts, allowing continued forward movement of the cell [25]. It was reported previously that cells that cannot migrate due to the inability to retract the trailing edge adopt a markedly elongated shape [25]. We observed this elongation in approximately 70% of carbachol-treated cells (Fig. 6B). This effect was observed in all experiments assessing cell migration (eight independent experiments). In contrast, PMA-treated cells did not exhibit this elongated morphology (Fig. 6C). This finding is consistent with a diminished ability of carbachol-treated cells to retract their trailing edges and move forward.

To investigate the effects of mAChR activation on cell detachment, we tested the ability of cells to withstand cell-substratum detachment induced by EGTA and EDTA. These agents chelate Ca^{2+} and Mg^{2+}, which are required co-factors for integrin-mediated adhesion (reviewed in [29]). Thus, EGTA/EDTA treatment induces cell detachment. We measured the percent of CHO-m3 cells detached from the substratum by exposure to EGTA/EDTA for 3 min. Carbachol treatment significantly diminished EGTA/EDTA-mediated detachment compared with control cells (Fig. 7). In contrast, PMA treatment did not elicit this response (Fig. 7).

We also studied the effect of the PKC inhibitors on EGTA/EDTA-induced cell detachment. The carbachol-mediated diminution of detachment was preserved in the presence of Go6976, which inhibits conventional PKC isoforms (Fig. 7). However, the effect of carbachol on detachment was not significant in the presence of either calphostin C or chelerythrine, which inhibited both conventional and novel PKC isoforms (Fig. 7). These findings suggest that novel PKC isoforms participate in the inhibition of cell detachment by carbachol.

4. Discussion

This study shows that M_1 and M_3 mAChR have a unique ability to diminish cell migration. This result is in surprising contrast to the effect of activating PKC with PMA, which increases cell migration. Our studies using PKC antagonists suggest that novel PKC isoforms may participate in the mAChR-mediated diminution of migration. In contrast, conventional PKC isoforms are likely involved in PMA-mediated migration. These findings suggest a unique mAChR-mediated signaling pathway that may be used by cells to diminish cell migration.

Previous studies indicate that the release of the cell-substratum bond at the rear of the cell is a crucial regulatory event in migration (reviewed in [25,26]). Significantly increasing integrin-mediated adhesion can inhibit CHO cell migration because the trailing edges of the cells cannot detach from the substratum [25,26]. These non-migratory cells adopt an elongated morphology due to the maintenance of integrin-ECM attachments at their trailing edges as they attempt to move forward [25]. M_3 mAChR activation may induce similar events in CHO-m3 cells. Activation of M_3 mAChR may alter integrin-ECM interactions in such a way that the cells cannot detach from the substratum and move forward. The elongated appearance

of the CHO-m3 cells following mAChR activation (Fig. 6 and [21,30]) is consistent with an inability of the cells to retract their trailing edges as they move forward.

It was reported previously that high concentrations of ECM proteins impair detachment of CHO cells from the substratum, and diminish cell migration [26]. Consistent with these results, we found that fibronectin concentrations of 5 μg/mL or greater inhibited the ability of PMA to stimulate cell migration. These high concentrations of fibronectin may impede the detachment of the PMA-treated cells, abolishing the stimulatory effects of PMA on migration. We also observed that carbachol inhibited migration regardless of the fibronectin concentration. This result may occur because mAChR activation inhibits the ability of the cells to detach from even low concentrations of fibronectin.

Carbachol and PMA may affect CHO-m3 migration differently because these agonists elicit different levels of integrin activity. Integrin activation induced by carbachol may be so intense or prolonged that the trailing edges of the cells cannot detach from the substratum. In contrast, integrin activation induced by PMA may be high enough to generate cell traction, but not high enough to inhibit cell-substratum detachment. According to this model, greater or more sustained integrin activity is generated by carbachol than by PMA in the CHO-m3 cells.

Several findings support the possibility that greater or more sustained integrin activity is induced by carbachol than by PMA. Stress fiber formation is induced more effectively by carbachol than by PMA in CHO-m3 cells [21]. Stress fiber formation depends upon active integrin-ECM attachments (reviewed in [31]). Thus, the superior ability of carbachol to induce stress fiber formation is consistent with carbachol inducing greater integrin activity. We also reported previously that the carbachol-induced spreading and elongation of CHO-m3 cells are maintained for 24 hr after exposure of the cells to carbachol [21]. In contrast, the PMA-induced spreading of CHO-m3 cells is more transient, and is lost within several hours after exposure of the cells to PMA [21,30]. The more sustained spreading and elongation of carbachol-treated cells compared with PMA-treated cells are consistent with more prolonged integrin activation induced by carbachol than by PMA. The results of our studies of cell detachment (Fig. 7) are also consistent with the model of greater integrin activation induced by carbachol than by PMA. Carbachol-treated cells detach less readily from fibronectin in the presence of EGTA/EDTA compared with PMA-treated cells. Chelation of divalent cations by EGTA/EDTA diminishes the ability of integrins to bind ECM proteins (reviewed in [29]). Thus, the resistance of carbachol-treated cells to the anti-adhesive effects of EGTA/EDTA is consistent with these cells having more stable integrin-ECM attachments than PMA-treated cells.

Activation of different PKC isoforms may contribute to the different effects of carbachol and PMA on the detachment and migration of CHO-m3 cells. This observation is based on our finding that the abilities of carbachol and PMA to alter cell behavior were affected differently by Go6976 and calphostin C. Conventional PKC isoforms may participate in the PMA-mediated stimulation of cell migration because this event is inhibited by Go6976, which selectively inactivates conventional PKC isoforms [23]. In contrast, novel PKC isoforms may participate in the mAChR-mediated inhibition of cell detachment and migration, because these events are inhibited by calphostin C, which antagonizes both novel and conventional PKC isoforms [24], but not by Go6976. Consistent with these possibilities, the novel PKC-δ isoform was reported previously to inhibit the migration of other cell types [32], whereas the conventional PKC-α isoform has been found to stimulate cell migration [33,34].

Calphostin C and Go6976 also have different effects on the carbachol-induced elongation of CHO-m3 cells [21]. Calphostin C causes carbachol-treated CHO-m3 cells to lose their elongated appearance [21]. This finding indicates that calphostin C may diminish carbachol-induced adhesion to the point that the cells no longer have impaired rear release. This possibility may explain why calphostin C abolishes the inhibitory effect of mAChR activation on cell migration. In contrast to the effects of calphostin C, Go6976 does not inhibit carbachol-induced cell elongation [21]. The inability of Go6976 to diminish carbachol-induced elongation is consistent with the inability of this antagonist to abolish the mAChR-mediated inhibition of cell migration.

Both PKC-δ and PKC-α are activated when CHO-m3 cells are exposed to either carbachol or PMA [21]. The ability of carbachol and PMA to activate the same PKC isoforms in CHO-m3 cells [21] indicates that simple activation of these isoforms cannot account for the different effects of carbachol and PMA on cell behavior. However, the abilities of these agonists to induce different kinetics of PKC activation [21] may contribute to the different effects of carbachol and PMA on the cells. Effectors other than PKC that are activated by carbachol but not by PMA undoubtedly also contribute to the inhibitory effects of carbachol on cell detachment and migration. The different migratory rates of cells treated with carbachol, PMA, or the PKC antagonists also probably result from several factors in addition to altered cell adhesion and detachment. This probability is indicated by the responses of chelerythrine-treated cells, which exhibited drastically reduced cell migration (Fig. 2), but little change in cell detachment (Fig. 7). We are currently investigating additional mAChR- and PKC-induced signals that alter cell migration.

The ability of the M_1 and M_3 mAChR to inhibit migration is a unique characteristic of these receptors. Activation of heterotrimeric G protein-coupled receptors generally has a stimulatory, rather than an inhibitory effect on cell migration. The ability to induce cell migration is more

commonly associated with G_i-coupled receptors, rather than G_q-coupled receptors [5,10,11]. For example, activation of transfected G_i-coupled opioid receptors stimulates the migration of HEK-293 [11] and pre-B [10] cells. In contrast, activation of transfected G_q-coupled M_3 mAChR does not detectably alter the migration of HEK-293 [11] or pre-B [10] cells. It is possible that M_3 mAChR activation can inhibit the migration of HEK-293 and pre-B cells, but the basal migration of these cells may be so slow that mAChR-mediated reductions in cell migration were not detected in previous studies [10,11].

Characterization of the multiple events involved in cell migration is of clinical importance. Migration is involved in many physiologic and pathophysiologic processes, including such apparently diverse entities as immune function, neuronal patterning, wound healing, and cancer metastasis. Inhibition of cell migration induced by M_3 mAChR activation may contribute to some of these processes. For example, it is intriguing to speculate that the activation of neuronal M_3 mAChR with the neurotransmitter acetylcholine may inhibit neuronal migration and thus contribute to neuronal patterning. Our results suggest that novel PKC isoforms may participate in the inhibition of cell migration induced by M_3 mAChR activation. These results define a unique pathway involved in the inhibition of cell migration. The identification of this pathway has potential import for the design of therapeutic strategies for regulating cell migration.

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