Differential Coupling of M₁ Muscarinic and α7 Nicotinic Receptors to Inhibition of Pemphigus Acantholysis*

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The mechanisms mediating and regulating assembly and disassembly of intercellular junctions is a subject of intensive research. The IgG autoantibodies produced in patients with the immunoblotting skin disease pemphigus vulgaris (PV) can induce keratinocyte (KC) dyshesion (acantholysis) via mechanisms that involve signaling kinases targeting intercellular adhesion molecules, thus providing a useful model to study the physiologic regulation of KC cohesion. Previous studies showed that activation of Src and protein kinase C are the earliest events in the PV IgG-induced intracellular phosphorylation cascades and that cholinergic agonists are effective for treating patients with pemphigus. In this study, we sought to elucidate the molecular mechanisms allowing cholinergic agonists to inhibit PV IgG-induced acantholysis and phosphorylation of KC adhesion molecules. The extent of acantholysis in KC monolayers correlated closely with the degree of PV IgG-induced phosphorylation of p120- and β-catenin, with classic isoforms of protein kinase C mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin. The M₁ muscarinic agonist pilocarpine blocked phosphorylation of both catenins, which could be abolished by the M₁ antagonist MT7. The α7 nicotinic agonist AR-R17779 inhibited phosphorylation of P120-catenin. The α7 agonist methyllycaconitine abolished the effect of AR-R17779. Okadaic acid abrogated protective effects of agonists on phosphorylation of β-catenin, and pervanadate, on that of p120-catenin. Stimulation of KCs with pilocarpine significantly (p < 0.05) elevated both serine/threonine and tyrosine phosphatase activities in KCs. AR-R17779 both stimulated tyrosine phosphatase and decreased PV IgG-induced Src activity. Methyllycaconitine released Src activity in intact KCs and caused acantholysis. Thus, downstream signaling from M₁ abolished PV IgG-dependent catenin phosphorylation due to activation of both serine/threonine and tyrosine phosphatases, whereas α7 action involved both activation of tyrosine phosphatase and inhibition of Src. These findings identified novel paradigm of regulation of signaling kinases associated with cholinergic receptors and provided mechanistic explanation of therapeutic activity of cholinomimetics in PV patients.

The intercellular adhesion of epithelial cells is mediated by four types of junctions: tight (zonula occludens), adherens (zonula adherens), desmosomal (macula adherens), and gap junctions (macula communications). The mechanisms mediating and regulating assembly and disassembly of intercellular junctions is a subject of intensive research. Although functional components of adhesion complexes are well known, much less is known about the signaling mechanisms that initiate, sustain, and terminate cell cohesion. Phosphorylation and dephosphorylation of components of adhesion complexes are probably the most important regulatory events altering both their structural integrity and the adhesive capacity. The IgG autoantibodies produced in patients with the immunoblotting skin disease pemphigus vulgaris (PV) can induce detachment of keratinocytes (KCs) (acantholysis) via mechanisms that involve signaling kinases targeting intercellular adhesion molecules, thus providing a useful tool to study the physiologic regulation of keratinocyte cohesion. PV is an IgG autoantibody-mediated disease of skin and mucosa leading to progressive blistering and non-healing erosions. Therapy of patients with pemphigus relies on the long term use of systemic glucocorticosteroids in relatively large doses, which, although life saving, may cause severe side effects, including death. Thus, the knowledge of the pathophysiology of acantholysis converges with that of the physiology of keratinocyte cohesion.

Adherens junctions are assembled by classic cadherins, transmembrane proteins that form homodimers with other cadherin molecules on adjacent cells in a Ca²⁺-dependent manner. They interact with the submembrane plaque proteins β-catenin and γ-catenin (or plakoglobin) linked to the actin cytoskeleton via α-catenin directly, or indirectly via vinculin or α-actin, as well as with p120-catenin. Both β- and p120-catenins have been shown to participate in the pathways activated by cadherin engagement leading to stable adherens junction formation (1, 2). Furthermore, p120-catenin is thought to regulate cadherin clustering during formation of adherens junctions. Desmosomal junctions also assemble in a Ca²⁺-dependent manner. The core proteins are the desmosomal cadherins desmogleins (Dsgs) 1–4 and desmocollins 1–3 forming both homo- and heterotypic interactions extracellularly. Phosphorylation of cadherin (3–5), γ-catenin (6), desmoplakin (7, 2)

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Cholinergic Regulation of Cell Adhesion

8), and Dsg (9–11) is usually associated with a loss of adhesion. Some intercellular junction proteins are phosphorylated on serine, some on tyrosine, and some on both residues. The phosphorylation state of a given adhesion molecule is determined by both protein kinase and phosphatase activities. Experimental inhibition of either tyrosine- or serine/threonine-specific protein phosphatases in both cases results in major changes of cell morphology manifested by rapid rounding-up of individual cells and alterations of monolayer integrity (3, 12).

PV IgG causes KCs to shrink, lose intercellular junctions, and round up (reviewed in Ref. 13). We have previously demonstrated that PV IgG increases the level of phosphorylation of E-cadherin by 42%, β-catenin by 37%, γ-catenin by 136%, and Dsg 3 by 300% (14). Acantholysis caused by pemphigus antibodies can be blocked by inhibitors of protein kinase C (PKC), Src, p38 mitogen-activated protein kinase, epidermal growth factor receptor (EGFR) kinase, other tyrosine kinases and calmodulin (15–17). Activation of EGFR due to binding of PV IgG to KCs is followed by phosphorylation of its downstream substrates, the MAPK ERK and the transcription factor c-Jun (18). Although both Src and EGFR kinases contribute to cell shrinkage and keratin filament aggregation, the peak of Src activity precedes that of EGFR kinase (17), indicating that engagement of Src/EGFR may be a key step that relays the signal emanating from interaction of PV IgG with various self-antigens on the keratinocyte plasma membrane to the pathway affecting adhesion complexes. Activation of PKC is also one of the earliest events of PV IgG-induced acantholysis (19). On the cell membrane of KCs, PV IgG binds to the Dsg antigens as well as other adhesion and non-adhesion molecules, including acetylcholine (ACh) receptors (reviewed in Ref. 20). Recently, it has been demonstrated that gene silencing of Dsg 1 and/or Dsg 3 affects the ability of KCs neither to maintain a polygonal shape and form intercellular junctions nor respond to PV IgG by elevation of the activities of Src and EGFR kinase (17). This suggested that Dsg-dependent signaling is a secondary, or tertiary, pathway mediating processing and utilization of internalized Dsg molecules rather than a primary downstream signaling emanating from the cell membrane of KCs due to PV IgG binding.

The network regulating phosphorylation of intercellular junction proteins is rather complex and interdependent with pathways mediating cell response to various kinds of environmental stimuli downstream of growth factors receptors, G-protein-coupled metabotropic receptors, and ion channels/pumps (21–23). ACh and related compounds can alter the assembly/disassembly of intercellular junctions and cell shape and motility of KCs in an autocrine/paracrine manner (reviewed in Ref. 24). ACh is remarkably abundant in the epidermis and other types of the surface epithelium (25, 26). ACh and related compounds affect keratinocyte function through binding to different classes of ACh receptors, the muscarinic (mAChR) and the nicotinic (nAChR) classes of cholinergic receptors (reviewed in Ref. 27). Human KCs express five, M1–M5, mAChR subtypes and α3, α5, α7, α9, α10, β1, β2, and β4 nAChR subunits. Both muscarinic and nicotinic agonists can increase the permeability of keratinocyte monolayers, cause acantholysis in vitro, and widen intercellular spaces in the epidermis. In contrast to antagonists, cholinergic agonists stimulate adhesion of KCs (reviewed in Ref. 24). The agonists can reverse acantholysis induced in keratinocyte cultures by cholinergic antagonists, the serine proteinase trypsin, and the calcium chelator EDTA (28).

Recent research convincingly demonstrated that the molecular mechanism of cholinergic control of keratinocyte adhesion involves an ACh receptor-dependent changes in the phosphorylation status of keratinocyte adhesion molecules. The pan-muscarinic antagonist atropine increased the phosphorylation level of E-cadherin by ~120%, Dsg 3 by 33%, and that of β- and γ-catenin by 50%, whereas the antagonist of ganglionic-type nAChRs mecamylamine produced only minor changes in the phosphorylation status of keratinocyte adhesion molecules (29, 30). In the presence of carbachol, a mixed muscarinic and nicotinic agonist and acetylcholinesterase inhibitor, atropine-induced phosphorylation was suppressed. Carbachol also treated PV IgG-induced acantholysis in culture of human KCs and epidermis of the neonatal mice that received PV autoantibodies (28, 31). The cholinomimetic drugs pyridostigmine bromide, an acetylcholinesterase inhibitor, and pilocarpine, a preferential M1 mAChR agonist (32), both showed therapeutic efficacy in recent clinical trials involving PV patients (33, 34). An indirect finding that cholinomimetics may be an effective treatment of pemphigus was provided by studies showing successful use of nicotinamide as a steroid-sparing agent in pemphigus (35). Nicotinamide exhibits cholinomimetic effects (36) due to both stimulation of ACh release (37) and inhibition of acetylcholinesterase (38). Finally, because cigarette smoke contains the cholinomimetic agent nicotine, a reported case of PV that improved due to cigarette smoking (39) further suggested that pharmacologic activation of the keratinocyte ACh axis can facilitate healing of pemphigus erosions. The therapeutic effects of cholinomimetics in PV may stem from their ability to abrogate PV IgG-induced phosphorylation of keratinocyte adhesion molecules, because carbachol and pyridostigmine bromide can decrease by severalfold the PV IgG-dependent phosphorylation of E-cadherin and γ-catenin (31). Thus, elucidation of the cholinergic control of keratinocyte adhesion merits further consideration because of a potential for development of novel anti-acantholytic therapies using cholinergic drugs.

In this study, we sought to elucidate molecular mechanisms allowing cholinergic agonists to inhibit PV IgG-induced acantholysis and phosphorylation of keratinocyte adhesion molecules. We found that PV IgG effects on KCs involved phosphorylation of both p120- and β-catenins, with PKC mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin. Downstream signaling from keratinocyte M1 mAChR interfered with PV IgG-dependent phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin. Downstream signaling from keratinocyte M1 mAChR interfered with PV IgG-dependent phosphorylation of both p120- and β-catenins, with PKC mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin. Downstream signaling from keratinocyte M1 mAChR interfered with PV IgG-dependent phosphorylation of both p120- and β-catenins, with PKC mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin. Downstream signaling from keratinocyte M1 mAChR interfered with PV IgG-dependent phosphorylation of both p120- and β-catenins, with PKC mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin. Downstream signaling from keratinocyte M1 mAChR interfered with PV IgG-dependent phosphorylation of both p120- and β-catenins, with PKC mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin.
MATERIALS AND METHODS

IgG Fractions and Reagents—The results reported herein were obtained in experiments utilizing pooled IgG fractions isolated by fast protein liquid chromatography protein G affinity chromatography from sera of six patients with mucocutaneous PV characterized elsewhere (17), and serum of healthy people was purchased from Sigma-Aldrich. The potent, M₁-selective inhibitor MT7 (also known as M₁-toxin 1 (40, 41)) was purified from the venom of Dendroaspis angusticeps as described elsewhere (42). The α7-prefering agonist AR-R17779 was synthesized at AstraZeneca Pharmaceuticals (Wilmington, DE). Both the preferential M₁, mACHR agonist pilocarpine and the preferential α7 nAChR antagonist methyllycaconitine (MLA) were purchased from Sigma-Aldrich. The PKC inhibitors Gö-6976 and rottlerin, the inhibitor of Src family of protein-tyrosine kinase PP2, the preferential inhibitor of PP2A phosphatase okadaic acid (OA), and the PTP inhibitor sodium orthovanadate (converted to pervanadate by hydrogen peroxide) were obtained from Calbiochem-Novabiochem Corp. (EMD Biosciences, Inc., La Jolla, CA). The PKC inhibitor chelerythrine was from Axonova, LLC (San Diego, CA). The FACE™ c-Src kit was purchased from Active Motif (Carlsbad, CA). The 6,8-difluoro-4-methylumbelliferone (DiFMUP)-based assays for measurement of the activities of tyrosine phosphatase (R-22067) and serine/threonine phosphatases (R-33700, adjusted for measurement of the activities of tyrosine phosphatase PP2A measurement) were from Molecular Probes, Inc. (Eugene, OR). Mouse monoclonal antibodies to β-catenin and phosphoserine/threonine were purchased from BD Biosciences (San Jose, CA), to p120-catenin, from Sigma-Aldrich Corp. Inc., and to phosphotyrosine, from Abcam Inc. (Cambridge, MA).

Keratinocyte Exposure Experiments—Human keratinocyte cultures were started from normal neonatal foreskins (25). The cells were grown in 75-cm² flasks (Corning Glass Works, Corning, NY) in serum-free keratinocyte growth medium containing 5 mg/ml epidermal growth factor and 50 μg/ml bovine pituitary extract (Invitrogen) at 37 °C in a humid, 5% CO₂ incubator at 0.09 mM calcium. The keratinocyte cultures used in experiments were between the passages 2 and 4, ~80% confluent, grown from at least three different foreskin donors. The IgG fractions were diluted in culture medium and added to the monolayers at the final concentration of 1 mg/ml. Prior to exposures, some monolayers were pre-treated with pharmacologic inhibitors (see “Results”). The control monolayers were left intact. Sixteen hours prior to experiments, the concentration of Ca²⁺ in growth medium was increased to 1.2 mM. All monolayers were exposed to test agents at 37 °C and 5% CO₂ for different periods of time (see “Results”) and then used in the Src, PP2A, or PTP assays in accordance to manufacturer’s protocols.

Assay of Keratinocyte Cell-Cell Attachment—The effects of test agents on spreading of keratinocyte cytoplasm and formation of intercellular junctions were measured using the monolayer permeability assay (28, 30). Briefly, a confluent keratinocyte monolayer was formed in Transwell-COL (Costar, Cambridge, MA) chambers inserted into the 24-well tissue culture plates 2–3 days after KCs were seeded at a cell density of 1 × 10⁴/100 μl keratinocyte growth medium into the chambers and cultivated at 37 °C in humid atmosphere with 5% CO₂. Confluent monolayers were fed with keratinocyte growth medium-containing test agents. After 1 h of incubation, the permeability of the monolayer was measured by adding 100 μl keratinocyte growth medium containing [³H]thymidine (1 μCi/insert, 6.7 Ci/mmol, New England Nuclear, Boston, MA) to each culture. Five minutes later, 100-μl aliquots of solution containing [³H]thymidine were taken in triplicate from each lower chamber. The more the cells became detached from each other and rounded up, the more the tracer penetrated into the lower chamber through the porous membrane of the upper chamber, and the higher the permeability coefficient (PC) values were obtained: PC = (cpm in experimental culture/cpm in control culture) × 100.

Immunoprecipitation and Western Blot Assays—KCs were scraped from the bottom of the dishes and placed in lysis buffer (0.1 M NaCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.2% Nonidet P-40, 1 μg/ml aprotinin, 2 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride). Samples were homogenized, and protein concentration was determined. The anti-p120- or β-catenin antibody was added to cell lysate, and the preparation was gently rocked at 4 °C overnight. A protein G–agarose bead suspension from the Protein G immunoprecipitation kit (Sigma-Aldrich) was added, and the mixture was incubated at 4 °C for 2 h. Beads were collected by pulsing 5 s in a microcentrifuge at 14,000 rpm and rinsed three times with ice-cold cell lysis buffer. The agarose beads were resuspended in SDS-sample buffer (0.5 M Tris-HCl, 10% SDS, 10% glycerol, 2.5% bromphenol blue, 5% β-mercaptoethanol). Protein samples were boiled and separated on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, blocked overnight with 1.5% bovine serum albumin in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 8.0), and incubated for 1 h with either phosphoserine (for β-catenin) or phosphotyrosine (for p120-catenin) antibody. Control blots stained with either anti-p120- or β-catenin antibody were run to confirm equal loading in the precipitates of the protein in question. After washing in Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with a secondary antibody, and then developed using the ECL Plus chemiluminescence detection system (Amersham Biosciences). To visualize antibody binding, the membranes were scanned with a Storm™/FluorImager (Molecular Dynamics, Mountain View, CA) or Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantitation of bands was done using ImageQuant software (43). The signal was expressed as arbitrary densitometric units. All blots studied were within the linear range of exposure.

Statistical Analysis—All experiments were performed in triplicates, and the results were expressed as mean ± S.D. Statistical significance was determined using Student’s t test. Differences were deemed significant if the calculated p value was < 0.05.

RESULTS

The Protein Kinases Mediating PV IgG-induced Phosphorylation of Keratinocyte Catenins—PV IgG significantly (p < 0.05) elevated serine phosphorylation of β-catenin and tyrosine phosphorylation of p120-catenin (Fig. 1). Inactivation of PKC
with chelerythrine, 1 μM, prevented PV IgG-induced serine phosphorylation of β-catenin (Fig. 1). The Src inhibitor PP2, 30 μM, inhibited tyrosine phosphorylation of p120-catenin (Fig. 1). These results indicated that PV IgG effects on KCs involve phosphorylation of both p120- and β-catenin, with PKC mediating serine phosphorylation of β-catenin and Src-tyrosine phosphorylation of p120-catenin.

PKC Isoforms Preferentially Mediate PV IgG-induced Phosphorylation of β-Catenin—To elucidate which PKC isoforms serine phosphorylation of β-catenin KCs exposed to PV IgG, we used the PKC inhibitor Gö-6976, which selectively inhibits PKC-α and -β isozymes without affecting PKC-δ, -ε, and -γ isoforms, and rottlerin (Rtn), washed, and lysed, and cell lysates were immunoprecipitated with either β-catenin or p120-catenin antibody and blotted with either phosphoserine or phosphotyrosine antibody, respectively. The blots were analyzed by quantitative densitometry as described under "Materials and Methods." The numbers underneath the bands indicate the mean densitometry values from three experiments as -fold of intact KCs taken as 1, and that on tyrosine phosphorylation of p120-catenin, in the presence of the PTP inhibitor pervanadate, 20 μM (Fig. 1). Pervanadate also diminished the inhibitory effect of AR-R17779 on PV IgG-dependent rise in the phosphorylation level of p120-catenin (Fig. 1). These results indicated that downstream signaling from keratinocyte mAChRs interferes with PV IgG-dependent catenin phosphorylation due to AR-R17779 (45), 100 μM, abolished tyrosine phosphorylation of p120-catenin (Fig. 1) but had no effect on serine phosphorylation of β-catenin (data not shown). The α7 antagonist MLA (46, 47), 10 μM, reduced the protective effect of AR-R17779 on tyrosine phosphorylation of p120-catenin (Fig. 1).

Roles of Phosphatases in Cholinergic Receptor-mediated Protection of KCs from PV IgG-dependent Catenin Phosphorylation—The protective effect of pilocarpine on PV IgG-dependent serine phosphorylation of β-catenin was decreased in the presence of 50 nm OA, the preferential inhibitor of PP2A (Fig. 1), and that on tyrosine phosphorylation of p120-catenin, in the presence of the PTP inhibitor pervanadate, 20 μM (Fig. 1). Pervanadate also diminished the inhibitory effect of AR-R17779 on PV IgG-dependent rise in the phosphorylation level of p120-catenin (Fig. 1). These results indicated that downstream signaling from keratinocyte mAChRs interferes with PV IgG-dependent catenin phosphorylation due to AR-R17779 (45), 100 μM, abolished tyrosine phosphorylation of p120-catenin (Fig. 1) but had no effect on serine phosphorylation of β-catenin (data not shown). The α7 antagonist MLA (46, 47), 10 μM, reduced the protective effect of AR-R17779 on tyrosine phosphorylation of p120-catenin (Fig. 1).

Alterations of Catenin Phosphorylation Correlate with Changes in Keratinocyte Cohesion—The biological significance of the results obtained in the phosphorylation experiments (Fig. 1) was investigated by measuring the corresponding changes in keratinocyte cohesion, using a quantitative assay of monolayer permeability. In agreement with previous findings (28, 48), PV IgG produced a multifold increase in the permeability of keratinocyte monolayers (acantholysis), whereas the effect of normal IgG was just as opposite (Fig. 2). Both AR-R17779 and pilocarpine efficiently blocked PV IgG-induced acantholysis (45), 100 μM, abolished tyrosine phosphorylation of p120-catenin (Fig. 1) but had no effect on serine phosphorylation of β-catenin (data not shown). The α7 antagonist MLA (46, 47), 10 μM, reduced the protective effect of AR-R17779 on tyrosine phosphorylation of p120-catenin (Fig. 1).

Cholinergic Agonists Abolish PV IgG-dependent Activation of Protein Kinases in KCs—The M1, mAChR-prefering agonist pilocarpine, 50 μM, efficiently blocked PV IgG-induced serine phosphorylation of β-catenin and tyrosine phosphorylation of p120-catenin (Fig. 1). The receptor-mediated mechanism of pilocarpine action was confirmed by the ability of the specific M1 blocker MT7 (40, 44) to reduce the protective effects of pilocarpine on phosphorylation of p120- and β-catenins (Fig. 1). The α7 nACHR-prefering agonist activation of both PP2A and PTP, and that the mechanism of action of AR-R17779 involves PTP, but not PP2A.

FIGURE 1. Pharmacologic interference with PV IgG-dependent phosphorylation of p120- and β-catenins. Keratinocyte monolayers were exposed for 90 min at 37 °C and 5% CO2, to 1 mg/ml of normal IgG or PV IgG, given alone or in combination with the test compounds 1 μM chelerythrine (Chl), 30 μM PP2, 50 μM pilocarpine (Pln), 100 μM AR-R17779, 30 μM MT7, 10 μM MLA, 50 μM OA, 20 μM pervanadate (Pnd), 1 μM G6-6976 or 5 μM rottlerin (Rtn), washed, and lysed, and cell lysates were immunoprecipitated with either β-catenin or p120-catenin antibody and blotted with either phosphoserine or phosphotyrosine antibody, respectively. The blots were analyzed by quantitative densitometry as described under "Materials and Methods." The numbers underneath the bands indicate the mean densitometry values from three experiments as -fold of intact KCs taken as 1.

Changes in Keratinocyte Cohesion—Using a quantitative assay of monolayer permeability, we measured the effects of pilocarpine and AR-R17779 on the activities of tyrosine and serine/threonine phosphatases. Stimulation of KCs with pilocarpine significantly (p <
0.05) elevated activities of both types of protein phosphatases (Fig. 3), which is in keeping with an earlier report that M1 can recruit protein phosphatase (50). The involvement of M1 mACHR was confirmed by the ability of MT7 to abolish the pilocarpine activity. The specificity of the results to activation of PP2A and PTP was demonstrated by blocking the effects with OA and pervanadate, respectively (Fig. 3). Whereas activation of a7 nACHR with AR-R17779 did not affect the PP2A activity in KCs, it significantly (p < 0.05) increased PTP activity, which could be abolished in the presence of MLA or pervanadate (Fig. 3). These results indicated that M1 inhibits PKC-dependent serine phosphorylation of β-catenins and tyrosine phosphorylation of p120-catenin via activation of PP2A and PTP, respectively. A lower efficacy of AR-R17779 toward PTP activation prompted a search for an alternative and/or additional mechanism of Src inhibition by this a7 agonist.

Inhibition of Src Activity via a7 nACHR—Because PV IgG up-regulates Src activity in KCs (17), and because Src can associate with a7 nACHR subunit (51), we asked if opening of this ACh-gated ion channel by AR-R17779 could affect previously activated Src. To test this intriguing possibility, we measured Src activity in KCs stimulated with PV IgG given alone or in combination with AR-R17779 with or without MLA. Treatment of KCs with AR-R17779 significantly (p < 0.05) decreased PV IgG-dependent Src activity, which could be abolished in the presence of MLA (Fig. 4). This observation indicated that a7 nACHR can directly inactivate Src in KCs.

Because keratinocyte a7 nACHR is constantly stimulated with endogenously produced and secreted ACh, and because the inhibitor of a7 nACHR MLA, just like PV IgG, altered keratinocyte cohesion (Fig. 2), we hypothesized that interruption of the physiologic signaling of ACh through a7 nACHR would release Src activity. As seen in Fig. 4, treatment of intact KCs with MLA significantly (p < 0.05) elevated Src activity. These findings indicated that constant stimulation of a7 nACHR in KCs by autocrine/paracrine ACh maintains Src in the inactive state and that interruption of ACh signaling releases Src activity, leading to catenin phosphorylation and weakening and/or loss of cell cohesion.

**DISCUSSION**

Results of this study demonstrated for the first time that PV IgG-induced acantholysis is associated with serine phosphorylation of β-catenins and tyrosine phosphorylation of p120-catenin. On the other hand, the mechanism of anti-acantholytic action of pilocarpine in PV patients involves activation of both PP2A and PTP, whereas stimulation of a7 nACHR in KCs activates PTP and inhibits Src. Thus, the protective action of cholinomimetics on PV IgG-induced acantholysis is apparently mediated by both activation of phosphatases and inhibition of Src family kinases. Fig. 5 shows a hypothetical scheme of cholinergic signaling that protects p120- and β-catenins from the PV IgG-in-
known that keratinocyte intercellular junctions are formed and maintained with participation of classic and desmosomal cadherins, such as E-cadherin and Dsg, respectively, as well as linker proteins, such as catenins. Phosphorylation of these and other adhesion molecules plays an important role in assembly/disassembly of intercellular junctions formed by KCs and other epithelial cells (52–56). Multiple regulatory mechanisms can control cellular homeostasis by regulating the levels of β-catenin. Serine phosphorylation of β-catenin decreases cell adhesion (57) and represents the initial event for its degradation (58). Although tyrosine phosphorylation of adherens junctions components is generally associated with disruption of cell-cell adhesion, the relationship between direct phosphorylation of p120-catenin and cadherin function has remained elusive and poorly understood (1). Results of the present study convincingly demonstrate that keratinocyte dyshesion correlate closely with the degree of tyrosine phosphorylation p120-catenin and serine phosphorylation of β-catenin.

PV autoantibodies provide a useful tool to study the physiologic control of keratinocyte cohesion, because they reproducibly induce acantholysis in vitro and in vivo and activate signaling pathways leading to phosphorylation and disabling of adhesion molecules (reviewed in Ref. 59). Previous studies have demonstrated that activation of Src (17) and PKC (19) is the earliest event in the PV IgG-induced phosphorylation cascades and that cholinergic agonists can treat pemphigus acantholysis by abolishing PV IgG-induced phosphorylation of adhesion molecules, thus improving the natural course of disease (reviewed in Ref. 24). The major questions posed by results of previous works are: 1) what ACh receptor subtypes are directly involved in the physiologic control of keratinocyte cohesion; 2) what adhesion molecules are the common targets for PV IgG and the ACh receptors regulating keratinocyte cohesion; and 3) what effector molecules (kinases and/or phosphatases) are engaged in the signaling pathways coupled by both PV IgG and ACh receptors in KCs? The results of the present studies partially answered these important questions and also helped resolve a controversy as to whether tyrosine phosphorylation of p120-catenin promotes or interferes with cell adhesion (1).

We have not overlooked possible effects of PV IgG on phosphorylation of Dsg 1 and 3. Although there were no previous data on Dsg 1 phosphorylation in pemphigus, we (14, 31) and others (11, 60) have reported an antibody-induced phosphorylation of Dsg 3. Hence, we started our studies with Dsg 1 and 3 phosphorylation assays. To match previously reported experimental conditions, we tested Dsg phosphorylation at several time points after exposure to PV IgG, i.e. 30, 120, and 180 min. We found practically no phosphorylation of Dsg 1 and 3 on the serine or tyrosine residues (data not shown). These results were not surprising. In previous works, measurable phosphorylation of Dsg 3 was demonstrated using the DJM-1 cutaneous squamous cell carcinoma cell line, which features high levels of phosphorylation of adhesion molecules and, therefore, is customarily used to study phosphorylation of keratinocyte adhesion molecules (61–64). In contrast to DJM-1 cells, PV IgG produces only minor changes in the level of phosphorylation of Dsg 3 in normal human KCs (11). The unidentified proteins of ~30 and 16 kDa found to be phosphorylated in the

produced phosphorylation and acantholysis. These results have salient clinical implications not only for treatment of patients with mucocutaneous blistering but also for therapeutic control of cancer metastases and wound epithelialization.

The mechanisms by which intercellular junctions assemble and disassemble in KCs are not fully understood. It is well

FIGURE 4. Effects on Src activity. The measurements of Src activity were performed as described under “Materials and Methods” 45 min after exposure of confluent keratinocyte monolayers to test agents at the concentrations described in the legend to Fig. 1. Asterisks, significant (p < 0.05) differences compared with control; #, significant (p < 0.05) differences compared with PV IgG given alone.

FIGURE 5. A hypothetical scheme of cholinergic signaling that protects p120- and β-catenins from the PV IgG-induced phosphorylation and KCs from acantholysis secondary to ligation of self-antigens (PV/AgS) on the cell membrane by autoantibodies.
cultures of normal human KCs treated with PV IgG by Berkowitz and co-authors (65) could not represent Dsg 1 or 3 because of the small size of phosphorylated molecules. Hence, we focused on normal KCs, rather than DJM-1 cells, because we needed a model system specific for both acantholytic action of pemphigus antibodies and anti-acantholytic action of cholinergic agonists. Unfortunately, this model does not allow accurate measurement of PV IgG-dependent Dsg 1 and 3 phosphorylation. Alternatively, Dsg phosphorylation is not a chief component of the pathophysiologic pathway leading to acantholysis in pemphigus. This fundamental question will be addressed in future studies.

The obtained results shed light on the mechanism of therapeutic effect of pilocarpine in patients with PV (34, 66). This M1 agonist ameliorated pemphigus acantholysis by inhibiting PKC-dependent serine phosphorylation of β-catenins and tyrosine phosphorylation of p120-catenin via activation of PP2A and PTP, respectively. Our findings are consistent with the following transduction cascade: M1 mAChR → G-protein → Ca2+ release → calmodulin → tyrosine phosphatase (67). Coupling of the M1 mAChR to PTP has been also reported by other workers (50). We have recently demonstrated that M1 mAChR synergizes with α7 nAChR to mediate cholinergic control of cell shape and motility of KCs (44). Cooperation between muscarinic and nicotinic pathways in KCs helps maintain polygonal shape and motility of KCs (44). Together, the ACh signaling axis in regulation and coordination of distinct events mediating assembly and disassembly of intercellular junctions in KCs, which warrants further investigation. Apparently, an interplay of the ACh receptor signals governs adhesion dynamics of KCs via an intrinsic level of fine-tuning of the homeostatic control of the signaling pathways that impact on the phosphorylation status and activity of adhesion molecules. This novel paradigm of regulation of the ACh receptor-associated signaling kinases and phosphatases is apparently exploited by cholinomimetic drugs that activate adhesion molecules by both inhibiting Src family kinases and activating protein phosphatases. Thus, novel therapeutic approaches to control mucocutaneous blistering, accelerate wound epithelialization, and prevent cancer metastases may be developed based on the anti-acantholytic effects of cholinomimetic drugs.

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