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Stimulation of the Nonneuronal Cholinergic System by Highly Diluted Acetylcholine in Keratinocytes

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Acetylcholine · Keratinocytes · High dilution · Sequential kinetic activation · Wound healing

Abstract
The physiological effects of acetylcholine on keratinocytes depend on the presence of nicotinic and muscarinic receptors. The role of nonneuronal acetylcholine in keratinocytes could have important clinical implications for patients with various skin disorders such as nonhealing wounds. In order to evaluate the efficacy of highly diluted acetylcholine solutions obtained by sequential kinetic activation, we aimed to investigate the effects of these solutions on normal human keratinocytes. Two different concentrations (10 fg/mL and 1 pg/mL) and formulations (kinetically activated and nonkinetically activated) of acetylcholine were used to verify keratinocyte viability, proliferation, and migration and the intracellular pathways involved using MTT, crystal violet, wound healing, and Western blot compared to 147 ng/mL acetylcholine. The activated formulations (1 pg/mL and 10 fg/mL) revealed a significant capacity to increase migration, cell viability, and cell proliferation compared to 147 ng/mL acetylcholine, and these effects were more evident after a single administration. Sequential kinetic activation resulted in a statistically significant decrease in reactive oxygen species production accompanied by an increase in mitochondrial membrane potential and a decrease in oxygen consumption compared to 147 ng/mL acetylcholine. The M1 muscarinic receptor was involved in these effects. Finally, the involvement of ERK/mitogen-activated protein kinases (MAPK) and KI67 confirmed the effectiveness of the single treatment on cell proliferation. The intracellular pathways of calcium were investigated as well. Our results indicate for the first time that highly diluted and kinetically activated acetylcholine seems to play an active role in an in vitro model of wound healing. Moreover, the administration of acetylcholine within the physiological range may not only be effective but is also likely to be safe.

Abbreviations used in this paper

Ach	acetylcholine
Chel	chelerythrine
KC	keratinocytes
MAPK	mitogen-activated protein kinases
NHEK	normal human epidermal keratinocytes
NO SKA	nonsequential kinetic activation
ROS	reactive oxygen species
SKA	sequential kinetic activation

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Introduction

Keratinocytes (KC) are the main component of the stratified epithelium enveloping the human body. KC functions, such as viability, proliferation, and migration, depend on a fine balance in control pathways in which the nonneuronal cholinergic system plays a major role [Kurzen et al., 2007]. This system acts by means of acetylcholine (Ach) and its nicotinic and muscarinic receptors via autocrine and paracrine mechanisms. Ach is an example of phylogenetically old molecules present in a wide range of living organisms ranging from prokaryotic to eukaryotic neuronal and nonneuronal cells. To date, studies on the biological role of Ach have mainly focused on its neurotransmitter function only, although this messenger is widely expressed and linked to basic nonneuronal cell functions [Wessler et al, 2003; Kurzen et al., 2007; Wessler and Kirkpatrick, 2008; Sato et al., 2010; Beckmann and Lips, 2013].

Ach is a crucial regulatory molecule for the skin since it has been demonstrated that normal human KC are able to synthesize, secrete, and degrade it [Grando et al., 1993]. The physiologic regulation of KC exerted by Ach depends on the presence of both types of cholinergic receptors, i.e., nicotinic and muscarinic, and on competitive or synergic interactions with one another. Hence, a single cytotransmitter, either Ach or a cholinergic drug, may exert differential effects on KC at different stages of their maturation [Grando et al., 2006]. Several important functions, including control of cell viability, proliferation, adhesion, migration, and differentiation, have been also attributed to muscarinic receptors in KC [Beck et al., 2006]. Simultaneous stimulation of nicotinic and muscarinic receptors by Ach may be necessary to synchronize and balance ionic and metabolic events within cells. In KC, binding of Ach to the cell membrane simultaneously elicits several distinct biochemical events, the "biologic sum" of which, added to the effects of other hormonal and environmental stimuli, determines the change in cell behavior during epidermal turnover [Grando et al., 1993, 2006]. In recent years, a number of studies have clarified the molecular mechanisms underlying skin trophism in order to identify new therapeutic strategies for skin diseases. Furthermore, some evidence is available about the therapeutic capability of cholinomimetics or blockers in two important skin diseases, i.e., pemphigus or psoriatic lesions [Grando et al., 1993, 2006]. Thus, studying the role of nonnervous Ach in KC development and functions could have important clinical implications for patients with several skin disorders such as nonhealing wounds or immune and inflammatory diseases. In addition, under pathological conditions, tissue levels of Ach in skin biopsies are greatly increased [Wessler et al., 2003]. It was recently demonstrated in human KC that highly diluted carbachol is able to increase transcription of matrix metalloproteinase MMP-3 as well as several ligands of the epidermal growth factor family [Gariboldi et al., 2009], both important factors involved in epidermal cell proliferation and wound repair. However, in terms of a therapeutic application of Ach, it must be considered that this is a highly reactive molecule and consequently its use can lead to side effects [Beck et al., 2006]. A promising way to obtain a fine regulation of physiological mechanisms could be the use of highly diluted substances as been demonstrated by several studies available in the literature. For example, recently, evidence of the efficacy of a highly diluted form (defined low doses) of IL-12 in modulation of Th1 versus Th2 was demonstrated in an asthma preclinical model [Gariboldi et al., 2009], suggesting a novel therapeutic approach to diseases which involve a Th1/Th2 imbalance. Moreover, it has been observed that low doses of IL-12 modulate T-cell subpopulations in cultures of lung cancer cells. The same doses of IL-12 also promote inhibition of the proliferation of lung adenocarcinoma cells in vitro [D'Amico et al., 2012]. Also, these highly diluted forms have been obtained via a sequential kinetic activation (SKA) technique and these cytokines have shown capability to exert immunomodulatory action in colon cancer as well [Radice et al., 2014; Roberti et al., 2014]. Lastly, these highly diluted SKA cytokines have been demonstrated to have a significant beneficial effect in skin diseases both in patients and in cultured cells [Barygina et al., 2015; Radice et al., 2015].

Thus, the aim of this study is to evaluate the effects of highly diluted Ach solutions obtained by SKA on cultured normal human KC and on an in vitro incisional wound model in order to confirm the hypothesis that a highly diluted form (defined as "low dose") of Ach could be a greater physiological stimulus for KC viability and proliferation.

Materials and Methods

Preparation of Ach Solutions

All dilutions were prepared starting from a stock solution (0.001 mg/mL) of Ach (Sigma-Aldrich; Saint Louis, MO, USA) in 0.9% NaCl. Based on previous knowledge of activated blends [Brod and Khan, 1996; Avvakumov et al., 1999], Ach solutions were prepared at 2 different concentrations: 10 fg/mL and 1 pg/mL. Each concentration was prepared using or not using the SKA method. The activated solutions, following dilutions, were kinetically energized by a mechanically applied force via a standardized shaking process called SKA; the applied shaking procedure is char-

acterized by vertical shaking, a 10-cm motion range, and a shaking speed corresponding to 100 oscillations in 10 s. All solutions were prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy).

For each treatment, the volume of each solution was calculated by comparing the volume added to the sample treated with 147 ng/mL Ach (corresponding to a concentration of 1 μM).

Cell Culture

Normal human epidermal keratinocytes (NHEK) from neonatal foreskin were purchased from Lonza (Basel, Switzerland) and cultured in KBM medium (Lonza) containing KGM-2 (keratinocyte growth medium-2) growth supplements (Lonza) in an incubator at 37°C, 5% CO_2, and 95% humidity [Seo et al., 2012]. Experiments were conducted at passages 3–6.

Experimental Protocol

The cells (1×10^3) were plated on 96-well plates for the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test and crystal violet staining; 5×10^4 cells were plated on 24-well plates to analyze reactive oxygen species (ROS) production; 4×10^4 cells were plated in black 96-well plates to study oxygen consumption and mitochondrial membrane potential; wound healing assay and Western blot were performed in 6-well plates until confluence to analyze the intracellular pathways.

The cells were treated with different preparations of Ach [Ach SKA 10 fg/mL, Ach SKA 1 pg/mL, Ach nonsequential kinetic activation (NO SKA) 10 fg/mL, and Ach NO SKA 1 pg/mL] compared to 147 ng/mL Ach, a nonactivated form used only as a positive control at the concentration reported in the literature [Metzen et al., 2003]. The protocol of treatment was divided into 2 steps. In the first step, the cells were treated at T0, checked every 24 h, and maintained for 144 h (protocol A); in the second step, the cells were treated every 24 h, checked every 24 h, and maintained for 144 h (protocol B). We also tested in protocol A the involvement of muscarinic receptors and calcium using a specific antagonist, treating cells 30 min before stimulation; in particular, for muscarinic receptor 1 we used atropine (7.25 μg/mL; Sigma, Milan, Italy) [Kühne et al., 2015] and for calcium we used H89 (519 ng/mL, inhibitor of PKA kinase; Sigma) and chelerythrine (384 ng/mL, inhibitor of PKC kinase; Cayman Chemicals, Cabru, Milan, Italy) [Chernyavsky et al., 2009; Cappellano et al., 2013].

MTT Test

After each stimulation, the NHEK were washed with PBS 1× and incubated with DMEM without red phenol and FBS containing 1% MTT dye (MTT-Based In Vitro Toxicology Assay Kit; Sigma-Aldrich) for 2 h at 37°C and 5% CO_2 [Uberti et al., 2011]. Cell viability was determined by measuring absorbance using a spectrometer (VICTORX4 multilabel plate reader) at 570 nm with correction at 690 nm and calculated by comparing the results to control cells (100% viable).

Crystal Violet

After each treatment the cells were fixed with 1% glutaraldehyde (Sigma-Aldrich) for 15 min at room temperature, washed, and stained with 100 μL 0.1% aqueous crystal violet (Sigma-Aldrich) for 20 min at room temperature. One hundred microliters of 10% acetic acid were added to multiwell plates and mixed before reading the absorbance at 595 nm using a spectrometer (VICTORX4 multilabel plate reader). The estimated number was calculated by comparing the results to the control cells (control T0), examined on the first treatment, and the variation of the untreated cells, checked every 24 h (control), was also reported.

ROS Production

The rate of superoxide anion release was measured using a standard protocol based on reduction of cytochrome C [Uberti et al., 2014]. In both treated and untreated cells, 100 μL cytochrome C were added and in another sample 100 μL superoxide dismutase were also added for 30 min in an incubator (all substances were from Sigma-Aldrich). The absorbance in culture supernatants was measured at 550 nm using a spectrometer (VICTORX4 multilabel plate reader) and O_2 was expressed as the mean ± SD (%) of nanomoles per reduced cytochrome C per microgram of protein compared to the control [Sun et al., 2005].

Oxygen Consumption and Mitochondrial Membrane Potential

In living NHEK plated and treated as previously described, the oxygen consumption and mitochondrial membrane potential were immediately measured simultaneously following the manufacturer's instructions using an Oxygen Consumption/Mitomembrane Potential Dual Assay Kit (Cayman Chemical Company; Ann Arbor, MI, USA). The fluorescence of oxygen consumption was measured by excitation and emission wavelengths at 380 and 650 nm, respectively, and the membrane potential was measured using JC-1 red aggregates at an excitation/emission of 650/690 nm and green monomers at an excitation/emission of 485/535 nm in a fluorescence spectrometer (VICTORX4 multilabel plate reader). The results are expressed as means ± SD (%) compared to control cells.

Cell Scratch Wounding Assay

A scratch wound healing assay was performed as previously described [Liang et al., 2007] in confluent monolayer cells using a sterile p200 pipette tip. Afterwards, the cells were stimulated with different Ach preparations and physiological saline buffer in different protocols (A and B) and monitored every 24 h for 144 h. After each time point, repopulation of the wounded areas was observed under a phase contrast microscope (Leica, Germany). In addition, some experiments were performed following protocol A only to determine the effects of the blockers during wound healing. Using the ImageJ image-processing program, the size of the denuded area was determined at each time point from digital images taken at 6 different areas. The results are expressed as means ± SD (%) of migrated cells.

Western Blot

After the stimulation cells at confluence were lysed in ice with complete tablet buffer (Roche) supplemented with 2 mM sodium orthovanadate, 30 μg proteins from each lysate were loaded onto 10 or 5% SDS-PAGE gels, and PVDF (polyvinylidene difluoride) membranes (GE Healthcare Europe GmbH; Milan, Italy) were incubated overnight at 4°C with anti-KI67 (1:500; Santa Cruz, CA, USA), anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (p-ERK) (1:1,000; Euroclone, Milan, Italy), anti-p44/42 MAPK (ERK1/2) (1:1,000; Euroclone), anti-M1 receptor (1:250; Santa Cruz, CA, USA), anti-phospho-PKA (1:250; Santa Cruz, CA, USA), and anti PKC (1:250; Santa Cruz, CA, USA). Protein expression was normalized to the specific total protein (if possible) and verified through β-actin detection (1:5,000; Sigma-Aldrich) and expressed as a mean ± SD (%).

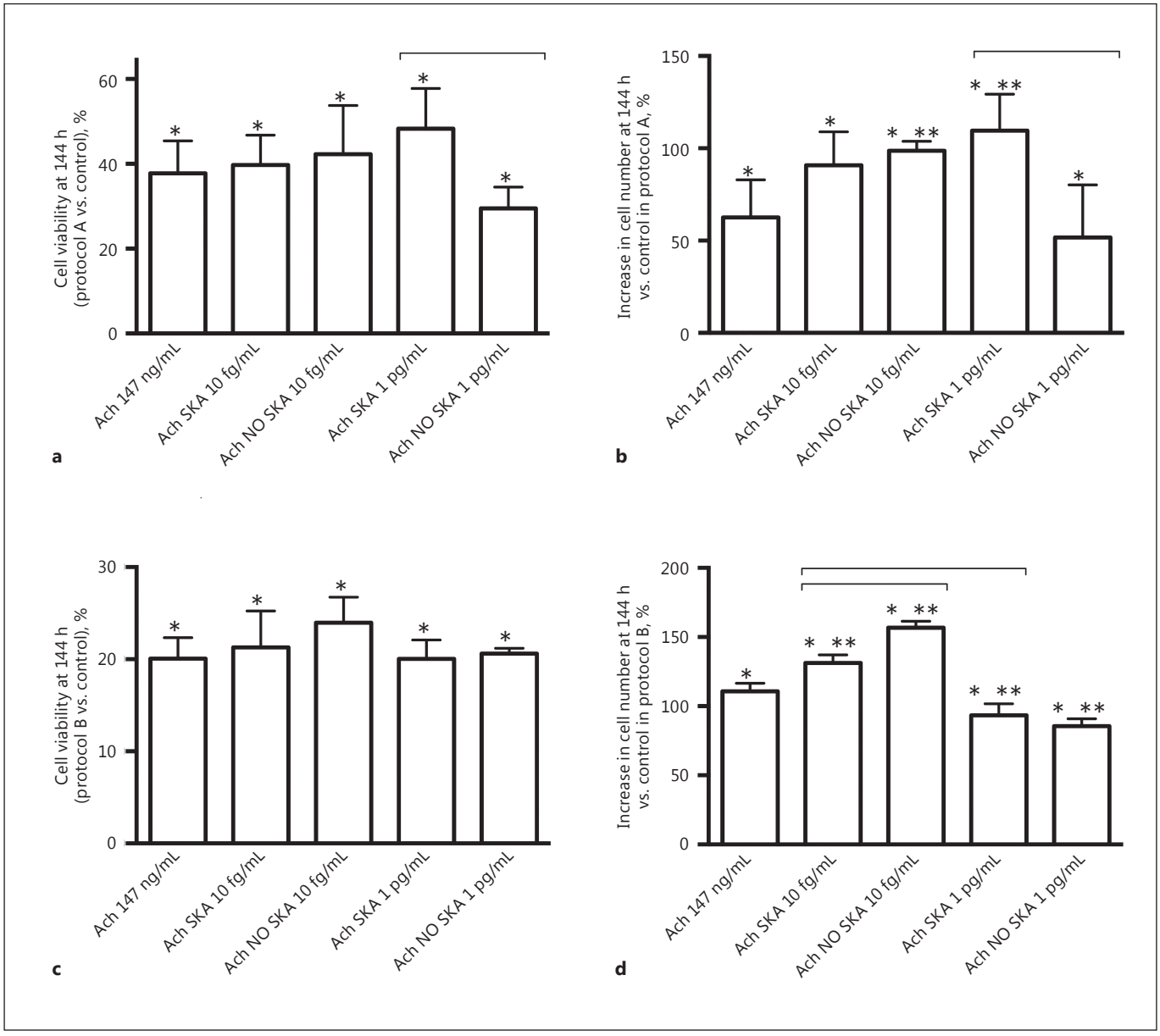

Fig. 1. Cell viability and cell counts after 144 h of stimulation with different formulations of Ach. **a**, **b** Results obtained by MTT and crystal violet staining in NHEK treated only at T0 with different formulations of highly diluted Ach and maintained for 144 h. **c**, **d** The same agents were added every 24 h for 144 h, and then NHEK cell viability and proliferation were measured. The results of the MTT test are expressed as means ± SD (%) of 6 biological replicates normalized to the control. In the crystal violet evaluation, cell counts are expressed as means ± SD (%) of 6 biological replicates. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. 147 ng/mL Ach. Bars indicate significance between the activated and nonactivated forms and between the 2 activated forms. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation.

Statistical Analysis

Results are expressed as means ± SD of at least 3 biological replicates for each experimental protocol, and each replicate was reproduced 3 times for each experimental protocol. Statistical comparisons between groups were made using one-way ANOVA with Bonferroni's post hoc test or the Mann-Whitney U test, as appropriate, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). $p < 0.05$ was considered statistically significant. All data from the densitometric analysis were normalized to control values (defined as 1). All other data from each experimental protocol were normalized to control values in percent (defined as 0%).

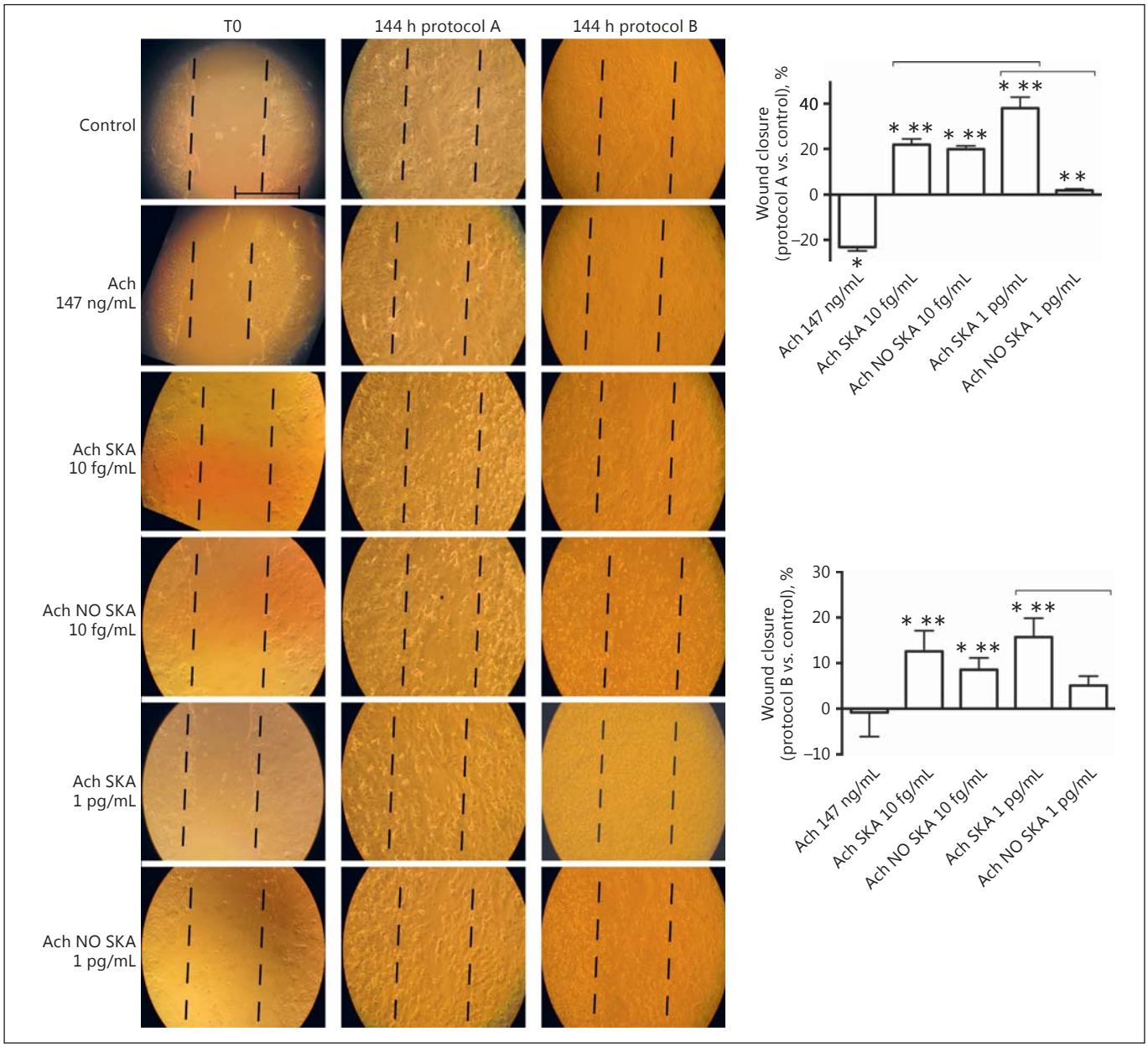

Fig. 2. Involvement of different formulations of Ach in NHEK wound closure. On the left are representative pictures of wound healing with each treatment at T0, after 144 h in protocol A, and after 144 h in protocol B taken through microscopy at an original magnification of ×20. Scale bar, 50 μm for all images. On the right, the wound closure area calculated by measuring the diminution of the wound bed surface by the time using ImageJ software in protocol A and B is shown. The results are expressed as means ± SD (%) of wound closure in 5 biological replicates normalized to each T0. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. 147 ng/mL Ach. Bars indicate significance between the activated and nonactivated forms and between the 2 activated forms. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation.

Results

Proliferation and Cell Viability

NHEK cells were treated using 2 protocols (i.e., A and B) for 144 h to verify the effects of highly diluted Ach compared to 147 ng/mL Ach on cell viability and proliferation.

As shown in Figure 1a and b, in protocol A, the formulation of sequentially kinetically activated Ach 1 pg/mL was able to improve cell viability (48.31 ± 9.4% compared to the control) compared to the nonactivated form (29.49

± 5.05% compared to the control), with 147 ng/mL Ach (37.8 ± 7.64% compared to the control) and with sequentially kinetically activated Ach 10 fg/mL (39.74 ± 7.01% compared to the control). This effect was statistically significant ($p < 0.05$) in comparison to untreated cells. This increase was also observed with crystal violet staining (Fig. 1b), in which the greater number of counted cells was observed with the sequentially kinetically activated form of Ach 1 pg/mL (109.6 ± 9.67% compared to the control). However, the sequentially kinetically activated solutions (Ach SKA 10 fg/mL and Ach SKA 1 pg/mL) were able to induce effects similar to those observed on cell proliferation using 147 ng/mL Ach (90.8 ± 8.11%; 109.6 ± 9.67% compared to the control vs. 62.61 ± 10.3% compared to the control). On the contrary, in protocol B (Fig. 1c, d) nonsignificant changes in cell viability and proliferation were observed in the presence of the sequentially kinetically activated form (Ach SKA 1 pg/mL and Ach SKA 10 fg/mL). The maximum effects were observed in the presence of nonsequentially kinetically activated Ach 10 fg/mL (NO SKA). These results demonstrate the importance of dilution and mechanical activation in the treatment of cells during the longest period.

Effects of Activated Ach Solutions on NHEK Migration

In this step, the effects of highly diluted Ach in different formulations on NHEK migration in a wound healing assay were evaluated (Fig. 2). Our results showed increased migration activity ($p < 0.05$) in KC treated with sequentially kinetically activated Ach 10 fg/mL and 1 pg/mL (both protocols A and B) compared to the control and 147 ng/mL Ach, and these effects were more evident in the presence of sequentially kinetically activated Ach 1 pg/mL (38 ± 4.92% in protocol A and 15.72 ± 4.12% in protocol B) after 144 h of treatment. The group treated with nonsequentially kinetically activated Ach 10 fg/mL in both protocols showed a greater effect than that treated with nonsequentially kinetically activated Ach 1 pg/mL, with a maximum effect in protocol A. On the contrary, nonsequentially kinetically activated Ach 1 pg/mL was effective in protocol B only. These data suggest that the sequentially kinetically activated solutions of Ach (10 fg/mL and 1 pg/mL) have an active role in wound closure, as shown in Figure 2 (left).

Effects of Highly Diluted Ach on ROS Production, Mitochondrial Membrane Potential, and Oxygen Consumption

In NHEK cells treated with 147 ng/mL Ach in both protocols an increase in ROS production of about 65.86 ± 7.69% (protocol A) and 67.5 ± 3.54% (protocol B) compared to the control was observed, but in the presence of sequentially kinetically activated Ach solutions (10 fg/mL and 1 pg/mL) this increase was significantly lower (13 ± 5.66 and 9.59 ± 2.65% in protocol A, respectively; 10 ± 1.41 and 8 ± 1.1% in protocol B, respectively) than what was observed with 147 ng/mL Ach and with the nonsequentially kinetically activated form in both protocols. The data are shown in Figure 3a and b. These data demonstrate the greater efficacy of the sequentially kinetically activated form in terms of normal KC health. As shown in Figure 3c and d, the activated solutions in both protocols (A and B) were able to increase ($p < 0.05$) the mitochondrial membrane potential compared to 147 ng/mL Ach and the nonsequentially kinetically activated form, whereas the maximum effects were observed in the presence of sequentially kinetically activated Ach 1 pg/mL (Ach SKA 1 pg/mL 79.01 ± 3.77% in protocol A and 81.6 ± 4.21% in protocol B compared to the control), indicating the greater biochemical energy of the cells. In order to obtain a more complete description of mitochondrial metabolism and function, an oxygen consumption assessment was also performed. As shown in Figure 3e and f, the rate of oxygen consumption decreased in all samples, indicating physiological activity of the mitochondria, and data were inversely proportional to mitochondrial potential movements ($p < 0.05$). It is noteworthy that the sequentially kinetically activated solutions (10 fg/mL and 1 pg/mL) induced oxygen consumption similar to that observed in samples treated with 147 ng/mL Ach (–18.76 ± 1.38 and –7.49 ± 2.5%, respectively, in protocol A; –3.3 ± 0.61 and –9.08 ± 0.88%, respectively, in protocol B),

Fig. 3. ROS production (**a**, **b**), mitochondrial potential membrane (**c**, **d**), and oxygen consumption (**e**, **f**) in NHEK cells. **a**, **b** ROS production is expressed as means ± SD (%) of 5 biological replicates normalized to control values and indicated as a percentage of cytochrome C reduced per microgram of protein with respect to the control. **c**, **d** Evaluation of the mitochondrial membrane potential in NHEK treated with different formulations of Ach; results are reported as means ± SD (%) of 5 biological replicates normalized to control values. **e**, **f** Oxygen consumption under the same conditions and treatments was assessed. The results are expressed as means ± SD (%). * $p < 0.05$ vs. control. ** $p < 0.05$ vs. 147 ng/mL Ach. Bars indicate significance between the activated and nonactivated forms and between the 2 activated forms. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation.

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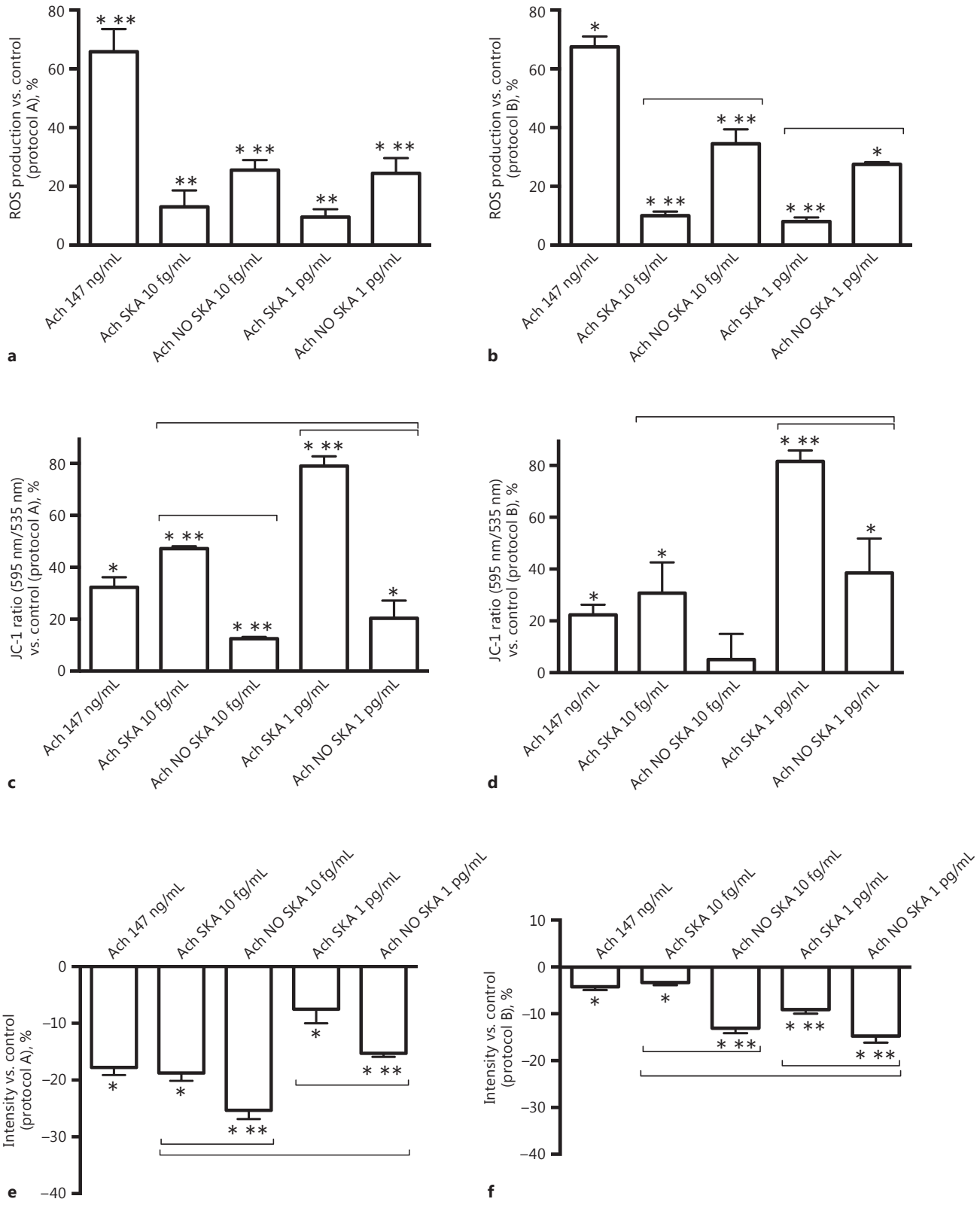

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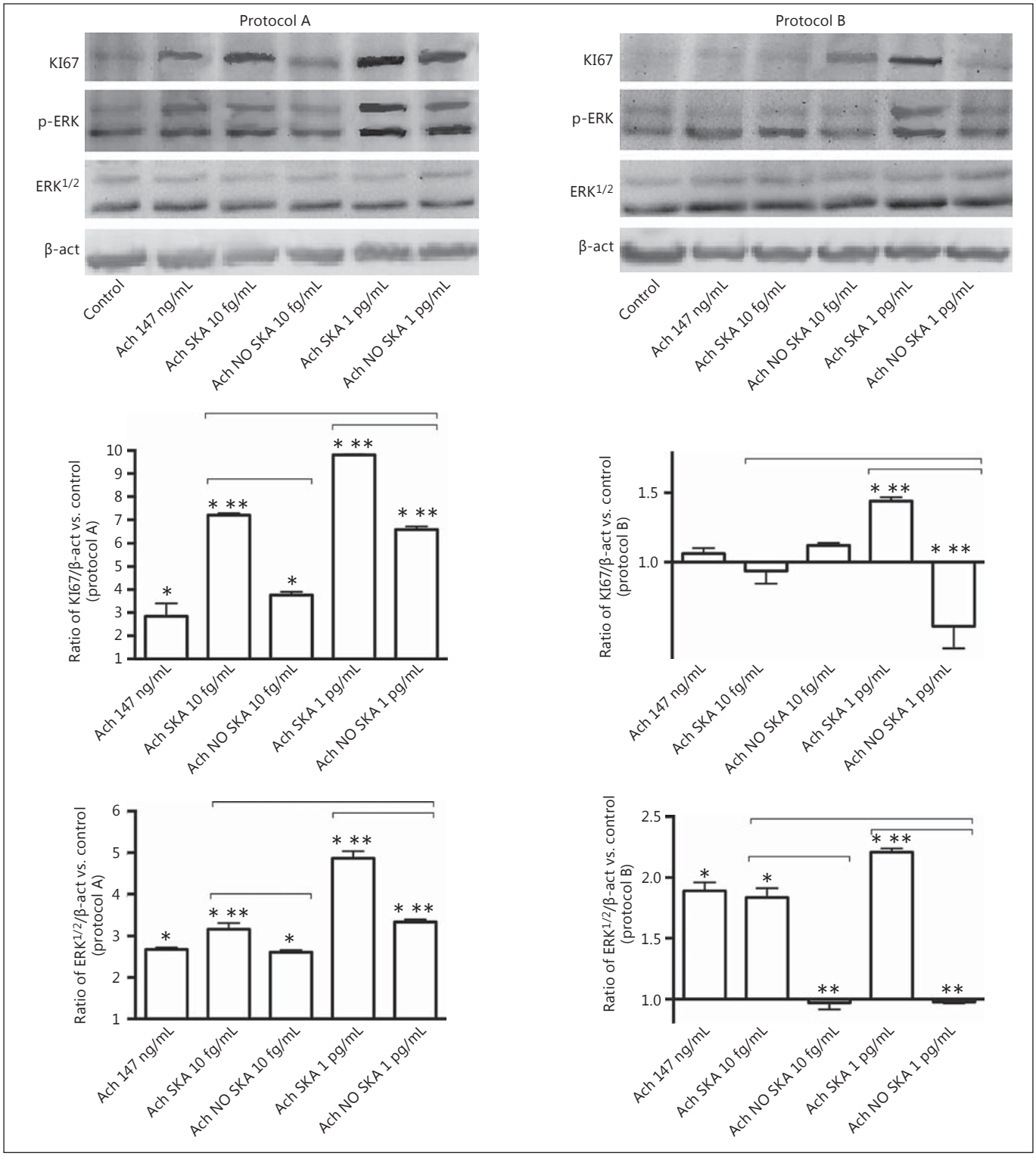

Fig. 4. Western blot and densitometric analysis of ERK/MAPK and KI67 in NHEK cells. Protein extracts were analyzed by immunoblotting with specific antibodies against the indicated proteins. The pictures represent 5 biological replicates for each experimental protocol (A and B). Data are expressed as means ± SD of 5 biological replicates for each experimental protocol (A and B). * $p <$ 0.05 vs. control. ** p < 0.05 vs. 147 ng/mL Ach. Bars indicate significance between the activated and nonactivated form and between the 2 activated forms. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation.

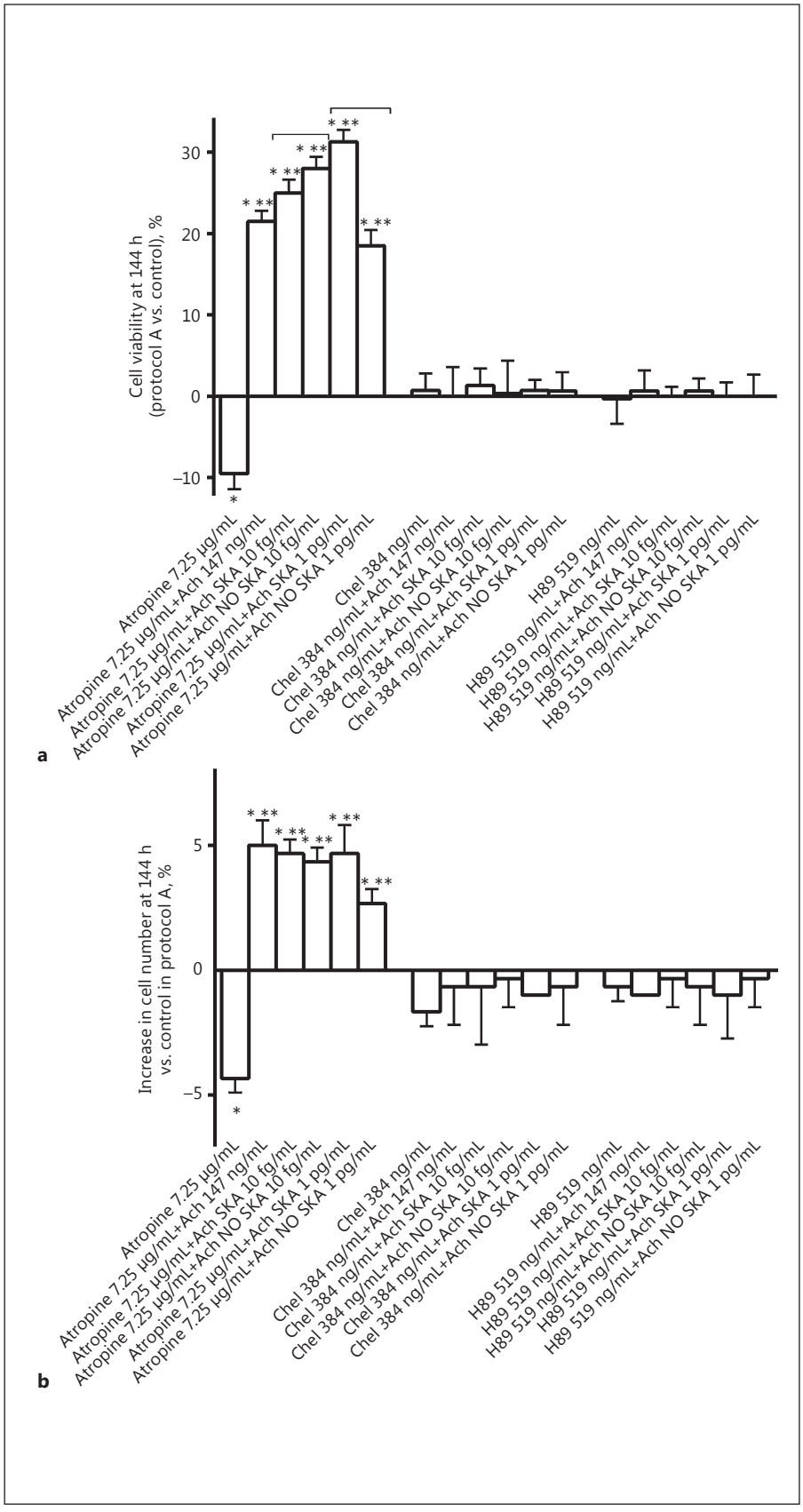

Fig. 5. Cell viability and proliferation in the presence of M1, PKC, and PKA blockers. Results obtained by MTT (**a**) and crystal violet staining (**b**) in NHEK treated only with protocol A. The results are expressed as a means ± SD (%) of 4 biological replicates normalized to the control. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. atropine 7.25 µg/mL. Bars indicate significance between the activated and nonactivated forms and between the 2 activated forms in pretreated samples. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation; Chel, chelerythrine.

whereas the nonactivated solutions showed a greater decrease in oxygen consumption, thus indicating possible stress conditions of the mitochondria.

Analysis of Intracellular Pathways

To investigate the mechanisms activated by highly diluted and 147 ng/mL Ach in cell proliferation, NHEK cells were treated with the same experimental protocols used before. As shown in Figure 4, all of these agents were able to induce significant changes in the expressions of KI67 and ERK/MAPK. In particular, we used the KI67 protein as a cellular marker to investigate KC proliferation as KI67 is known to be specifically expressed in nuclei of proliferating cells during the cell cycle. As reported in Western blot and densitometric analyses (Fig. 4), in both protocols sequentially kinetically activated Ach 1 pg/mL was able to induce a significant increase ($p < 0.05$) in KI67 compared to Ach 10 fg/mL and Ach 147 ng/mL, and these data confirmed the ability of sequentially kinetically activated Ach 1 pg/mL to improve cell proliferation in NHEK cells. However, Ach 10 fg/mL also showed a significant efficacy, in particular in protocol A, in which the increase in KI67 was higher compared to that with either 147 ng/mL Ach or the nonsequentially kinetically activated form. In addition, ERK, important members of the MAPK family regulating cell migration, were evaluated. In both protocols the activated solution of sequentially kinetically activated Ach 1 pg/mL was able to increase the expression of ERK1/2, and this effect was more evident in protocol A compared to what was observed with nonsequentially kinetically activated 147 ng/mL Ach and sequentially kinetically activated Ach 10 fg/mL. However, the sequentially kinetically activated Ach 10 fg/mL solution was more effective than the nonsequentially kinetically activated one in inducing phosphorylation of ERK/MAPK. This is an important finding since ERK phosphorylation acts as a switch for other intracellular pathways and thus is a clear index of cellular health. All of these data demonstrate the greater influence of the highly diluted Ach on cell proliferation of KC compared to the ponderal Ach concentration.

Analysis of the Involvement of Muscarinic Receptor Subtypes and Calcium Signaling in Effects Induced by Ach

Since the effects induced by Ach are mediated through its muscarinic and nicotinic receptors via the calcium signaling cascade, some of these elements were also analyzed during protocol A. In particular, the muscarinic receptor subtype 1 (M1) was analyzed. As shown in Figure 5, pretreatment with atropine at 7.25 μg/mL (M1 inhibitor) was able to reduce cell viability and proliferation of NHEK cells. In particular, in the presence of different formulations of Ach a significant reduction ($p < 0.05$) in the beneficial effects on cell viability and proliferation was observed and this effect was more evident in the presence of sequentially kinetically activated Ach 1 pg/mL. In particular, a decrease of about 40% in cell viability and about 97% in cell proliferation compared to stimulation without pretreatment was observed.

In addition, the importance of the involvement of the M1 receptor in the mechanism activated by all forms of Ach was also clarified by the experiments on wound healing, in which pretreatment with atropine at 7.25 μg/mL significantly reduced the area of closure alone and also in the presence of all forms of Ach (Fig. 6). Finally, as reported in Figure 7, sequentially kinetically activated Ach 1 pg/mL and 10 fg/mL were able to induce a significant increase (Ach SKA, $p < 0.05$) compared to the control and to the corresponding not-activated form in a manner similar to that observed with 147 ng/mL Ach. In order to verify this effect, NHEK cells were pretreated with atropine (7.25 μg/mL) alone and in the presence of the different formulations of Ach. This pretreatment prevented the activation of M1, and successive stimulation with different formulations of Ach was not able to restore this condition. These data confirm the involvement of muscarinic receptor in NHEK cells. The downstream signaling from the muscarinic and nicotinic classes of Ach receptors involves the second messenger pathways that control the expression and activity of effector molecules via the common signaling cascade Ca^{2+}-CamKII-PKC-MEK-ERK. In this context

Fig. 6. Involvement of M1, PKC, and PKA blockers in NHEK wound closure. **a–d** Representative pictures of wound healing with each treatment at T0 and after 144 h with protocol A through microscopy at an original magnification of ×20. Scale bar, 50 μm for all microscopy pictures. **e–g** Wound closure area; areas were calculated by measuring the decrease in the surface of the bed of the wound over time using the ImageJ software (protocol A). The results are expressed as means ± SD (%) of the wound closure of 4 biological replicates normalized to each T0. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. a specific blocker. Bars indicate significance between the activated and nonactivated forms and between the 2 activated forms in pretreated samples. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation; Chel, chelerythrine.

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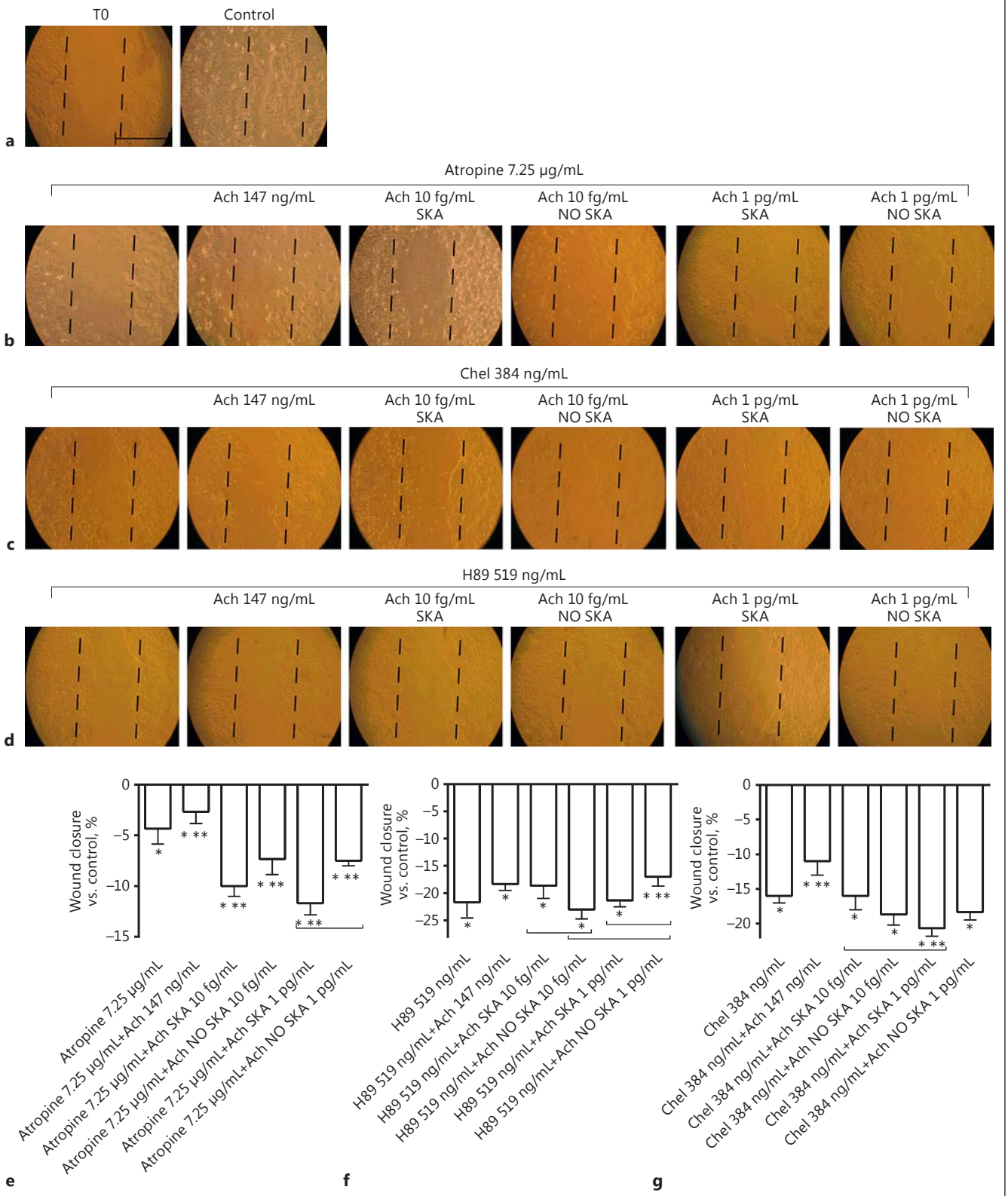

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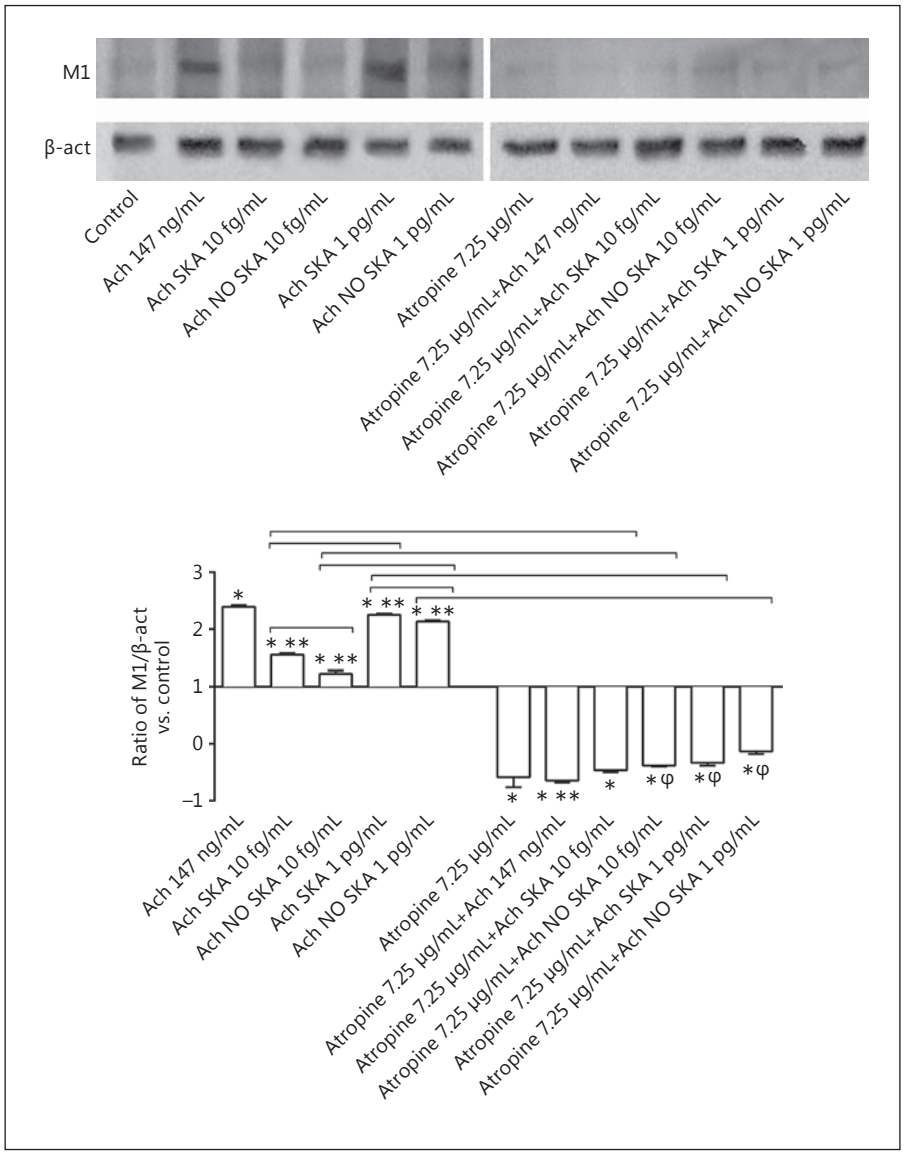

Fig. 7. Western blot and densitometric analysis of the M1 receptor in NHEK cells. Protein extracts were analyzed by immunoblotting with specific antibodies against the indicated proteins. The pictures represent an example of 3 biological replicates for protocol A. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation. Data are expressed as means ± SD of 3 biological replicates. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. 147 ng/mL Ach. $^{\varphi}$ $p < 0.05$ vs. 7.25 μg/mL atropine (specific M1 inhibitor). Bars indicate significance among the different forms of Ach, between activated forms alone and in the presence of pretreatment with atropine.

PKC and PKA kinases were also analyzed in NHEK cells treated under the same conditions as reported before. As shown in Figure 5, the specific blockers of PKC (chelerythrine at 384 ng/mL) and PKA (H89 at 519 ng/mL) were able to prevent any increase in cell viability or cell proliferation, maintaining the cell at a basal-like level. In the presence of all forms of sequentially kinetically activated and nonsequentially kinetically activated Ach, these effects were not reverted. These data confirm the ability of these blockers to modulate specific intracellular mechanisms as reported in the literature. In addition, similar data were also observed in wound healing experiments in which the blockers prevented the migration of cells and consequently closure of the lesion area. Finally, as shown in Figure 8, all of the forms (sequentially kinetically activated and nonsequentially kinetically activated) and 147 ng/mL Ach were able to induce the activation of PKC and PKA, indicating that Ach was able to involve the calcium intracellular pathway to exert its effects. In particular, 147 ng/mL Ach and sequentially kinetically activated Ach 10 fg/mL showed similar effects. However, all other formulations were able to induce the activation of these kinases ($p < 0.05$) compared to the control. These findings were confirmed by the pretreatments with chelerythrine (384 ng/mL) and H89 (519 ng/mL) for PKC and PKA,

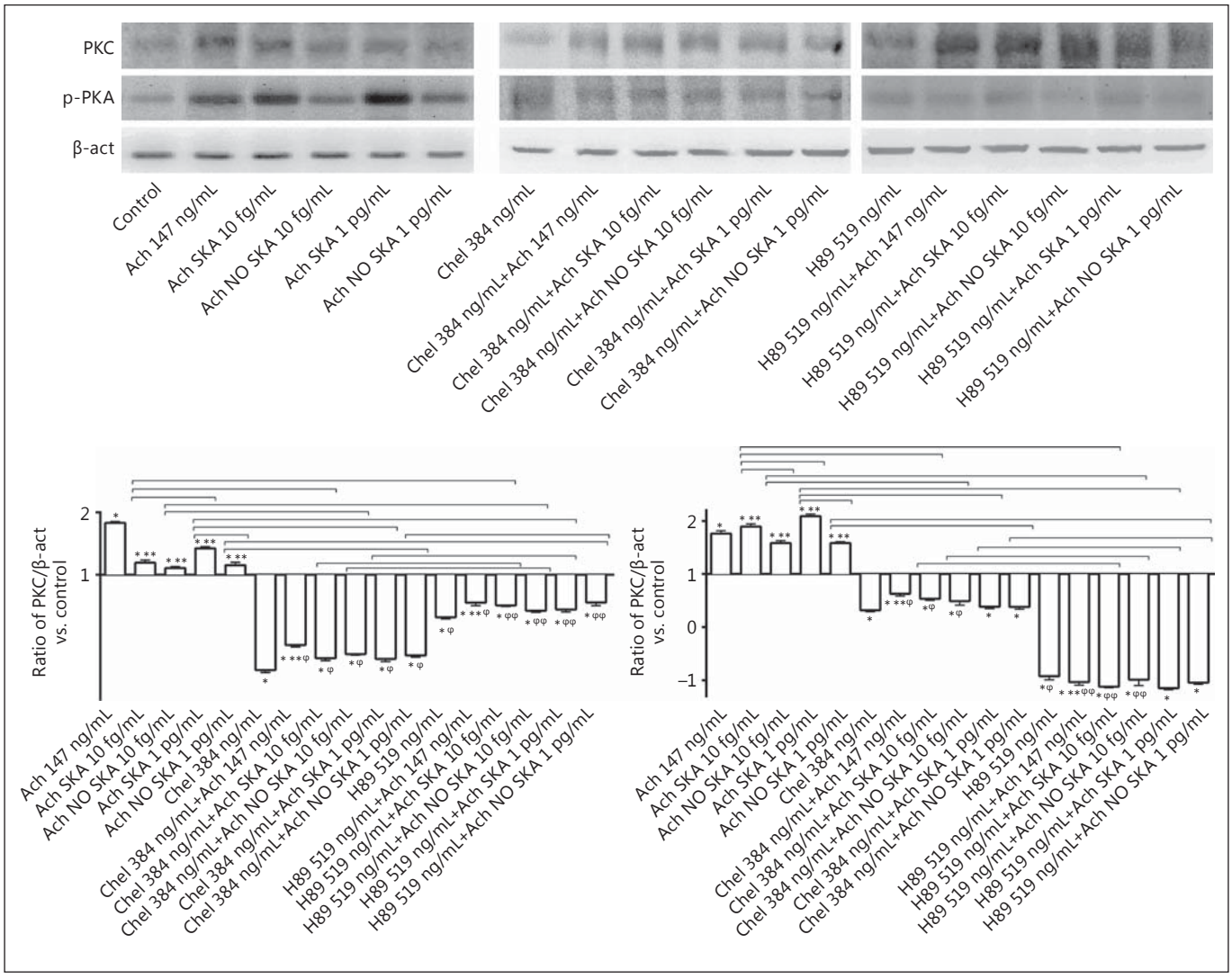

Fig. 8. Western blot and densitometric analysis of PKC and PKA in NHEK cells. Protein extracts were analyzed by immunoblotting with specific antibodies against the indicated proteins using also a specific antagonist (384 ng/mL chelerythrine and 519 ng/mL H89, respectively). The pictures represents an example of 3 biological replicates for protocol A. Data are expressed as means ± SD of 3 biological replicates. Ach, acetylcholine; SKA, sequential kinetic activation; NO SKA, nonsequential kinetic activation; Chel, chelerythrine. * $p < 0.05$ vs. control. ** $p < 0.05$ vs. 147 ng/mL Ach. $^{\varphi}$ $p < 0.05$ vs. 384 ng/mL chelerythrine. $^{\varphi\varphi}$ $p < 0.05$ vs. 519 ng/mL H89.Bars indicate significance among the different forms of Ach, between activated forms alone, and in the presence of pretreatment with specific blockers.

respectively. These agents were analyzed alone and in combination with the different formulations of Ach. As evidenced by Western blot and densitometric analysis in both of these conditions, stimulation with Ach was not effective in inducing activation of the specific kinase. These findings confirm the data observed with ERK phosphorylation and demonstrate the great influence of the highly diluted sequentially kinetically activated Ach on KC functions.

Discussion

Burn injuries, deep erosions, chronic ulcers, diabetic wounds, and graft donor sites are a few of the clinical situations [Chernyavsky et al., 2012] in which epithelialization is uncontrolled or incomplete. In this context, we decided to study novel approaches to facilitate epithelialization. In the last year, continuously increasing evidence for an extremely high sensitivity of biological objects to chemical

endogenous effects has been reported [Werkheiser et al., 2011; Marzotto et al., 2014; Landreneau et al., 2015]. Organs, tissues, and cells are capable of reacting to the presence of peptides, hormones, and molecular messengers at very low concentrations. A pioneering study demonstrated that low doses of solutions of mechanically activated IL-12 and IFN-γ, codelivered via the oral route to experimental asthmatic mice, can revert this pathological condition, restoring a normal balance between Th1- and Th2-derived cytokines and leading the animals to a healthy condition [Gariboldi et al., 2009]. Moreover, many clinical trials have tested highly diluted SKA drugs in various pathologies. Significant effectiveness has been reported in supportive therapy and pain relief in cancer patients [Rajendran, 2004; Sunila et al., 2009; Radice et al., 2014]. In addition, a reduction of symptoms and an improved quality of life have been observed in patients with psoriasis or vitiligo [Witt et al., 2009; Roberti et al., 2014; Barygina et al., 2015] or in immune system imbalances [Kho et al., 2012].

In this work, normal human epithelial KC were used to study the effects of highly diluted and mechanically activated Ach solutions (10^{-12}–10^{-14}) in an incisional wound model to confirm the hypothesis that low-dose Ach could be a more physiological stimulus for KC viability, proliferation, and migration.

Two different protocols have been used in order to assess the best modality of stimulation of cultured KC. Protocol A, characterized by a single stimulation, appears to be more effective than the protocol with repeated stimulations every 24 h (protocol B) on cell proliferation, viability, and migration. We investigated cell proliferation and cell migration in different experiments. These two biological functions were involved in two important physiological aspects of skin regeneration – during the creation of a wound the cells migrate and after its closure the cells return to a normal turnover. It can be hypothesized that a single stimulation triggers a sequence of intracellular reactions without any negative effect. Moreover, in both protocols low-dose Ach induced a lower ROS production than high-concentration Ach and the control.

One of the most compelling findings of this work is the difference in responses between sequentially kinetically activated and nonsequentially kinetically activated Ach solutions, with the former being much more effective than the latter. The observation of an increased effect induced by mechanical activation of an active principle is not completely new and has been observed by other authors [Gariboldi et al., 2009]. However, in the present work, enhanced effects due to mechanical activation have been demonstrated for the first time within the nonneuronal cholinergic system. The mechanisms underlying this increased effectiveness are still unclear. Here only a mere observation of the increased efficacy of sequentially kinetically activated versus nonsequentially kinetically activated low-dose Ach solutions is reported. More research will be necessary in the future in order to clarify this aspect.

Ach is present in all living cells [Hebb, 1962; Grando et al., 1993; Fania et al., 2012]. Human skin presents the highest concentration of free Ach [Klapproth et al., 1997; Fania et al., 2012], which depends on its production and degradation induced, respectively, by choline acetyltransferase and acetylcholinesterase. To exert its effects, Ach binds its muscarinic and nicotinic receptors (the muscarinic ones are transmembrane glycoproteins applying their function through G proteins and leading to activation of the second messengers; the nicotinic ones are ligand-gated ion channels that modify the flow of Na^+, Ca^{2+}, and K^+) [Fania et al., 2012]. The concentrations of Ach used in this study can be considered very similar to physiological release from human KC. Grando et al. [1993] reported that a single KC synthesizes a mean of 2×10^{-17} moles and releases 7×10^{-19} moles Ach/min. This fits very well with the amount of Ach administered to cultured cells during the experiments described in this work. Data available in the literature show significant improvements in mucocutaneous diseases in patients who received cholinergic agents as treatment for a concurrent disease or owing to the habitual use of nicotine products [Kuwahara et al., 2000; Mehta and Martin, 2000]. Those reports suggest that cholinergic agents have a huge potential for dermatological use.

Human KC possess cholinergic enzymes for Ach synthesis and degradation [Grando et al., 1993] and are also responsive to Ach, believed to act as a local hormone in the epidermis [Grando et al., 1993; Beck et al., 2006]. Ach acts via the calcium pathway, which funtions as a mediator of its effects on the epidermis [Grando et al., 2006] and then activates all of the intracellular pathways involved in calcium-signaling transduction, including ERK/MAPK [Beck et al., 2006; Kühne et al., 2015]. It is important to consider the difference in activity which we observed between 147 ng/mL Ach and sequentially kinetically activated or nonsequentially kinetically activated forms on proliferation, viability, cell migration, ROS production, oxygen consumption, and the mitochondrial membrane potential of NHEK. In both protocols (A and B), the sequentially kinetically activated Ach 1pg/ml formulation was able to improve cell viability compared to 147 ng/mL Ach and it induced effects similar to those of 147 ng/mL Ach on cell proliferation. In a minor manner, similar effects were also observed in the presence of sequentially kinetically acti-

vated Ach 10 fg/mL. Ach 147 ng/mL was chosen as the control because it was demonstrated, with a dose-response curve, to be the most effective concentration in a similar HaCaT (spontaneously transformed aneuploid immortal human keratinocyte cell line) experimental model [Metzger et al., 2005]. Data described in this work indicate for the first time the efficacy of activated low doses of Ach on cell culture and demonstrate a minor toxicity compared to 147 ng/mL. For this reason, low-dose Ach could be important for the development of new therapies for nonhealing wounds. As a matter of fact, nonhealing wounds are a major healthcare problem involving important social and economic aspects. Old age, immobility, and repeated trauma create conditions for the genesis and maintenance of inflammatory processes leading to wound formation. In addition, metabolic, neurologic, and circulatory factors play an important role in wound formation and contribute to chronicity. For this purpose, the ability of cells to migrate in the presence of the same agents and conditions used before was also investigated in a wound healing assay model. Sequentially kinetically activated forms (1 pg/mL and 10 fg/mL) revealed a greater capacity for migration compared to 147 ng/mL Ach, and these effects were more evident after a single administration. This is an important element because the response to doses of Ach may depend on its sensitivity to external perturbations. To verify these beneficial effects on wound closure under physiological conditions, ROS production, oxygen consumption, and mitochondrial membrane potential were investigated. Sequentially kinetically activated forms showed a statistically significant decrease in ROS production accompanied by an increase in mitochondrial membrane potential and decreased oxygen consumption in comparison with 147 ng/mL Ach, indicating the better biochemical energy of the cells. Also in this case, protocol A showed greater effects, indicating that a single treatment is enough to induce beneficial effects. Finally, the involvement of ERK/MAPK (involved in the regulation of cell migration) and KI67 (expressed in the nuclei of proliferating cells during the cell cycle) in the mechanism activated by Ach confirmed the better influence of the single treatment (protocol A) versus continuous treatment of the activated low doses on cell proliferation compared to 147 ng/mL Ach. Since protocol A showed the better efficacy, the other experiments on the M1 receptor and the calcium signaling cascade were performed only under this condition. Sequentially kinetically activated Ach 1 pg/mL confirmed its higher effectiveness compared to the sequentially kinetically activated Ach 10 fg/mL formulation in M1 receptor, PKC, and PKA activation. These findings confirm that highly diluted Ach acts through the same mechanisms as concentrated Ach and support the hypothesis of beneficial effects exerted by low doses without any negative effect (e.g., oxidative injury).

It is noteworthy that, although 147 ng/mL Ach showed some efficacy in cell proliferation, it may be harmful to the cells due to a significant increase in ROS production.

Our findings suggest that a single treatment of low doses (10^{-12}–10^{-14}) of sequentially kinetically activated Ach triggers a cascade of dynamic events that results in better regulation of KC functions, cell viability, proliferation, and migration. These elements are very important for hypothetical possible fields of application in the clinical setting.

In conclusion, low doses of sequentially kinetically activated Ach seem to play an active role in an in vitro model of wound healing. Moreover, these data suggest that administration of Ach at doses in a physiological range may not only be effective but is also likely to be safe. An intriguing aspect of this study is with regard to the difference in activity that was observed between sequentially kinetically activated and nonsequentially kinetically activated solutions, with the former being more effective than nonactivated solutions. The use of activated blends is common in different fields, including pharmaceutical technology, to obtain a high therapeutic potential with very low dosages.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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