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Voltage-activated Cl^- Conductance in Cultured Human Melanocytes

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Using the whole-cell patch-clamp technique we recorded Cl^- currents in cultured human melanocytes. With high symmetrical Cl^- solutions melanocytes expressed time-dependent Cl^- currents that did not run down for approximately 30 min. The currents showed voltage-dependent activation and reached a steady-state at about 500 msec during hyperpolarizing pulses. Current vs. voltage relation indicates that the current was activated by more hyperpolarizing voltage steps, showing inward rectification. Based on conductance vs. voltage relation the half activation voltage of the current was determined to be −60 mV. When tail current amplitudes were plotted as a function of test pulses, tail current reversed at −10 mV, close to ECl of 0 mV in this condition, indicating that the observed current was specific Cl^- current. (Ajou Med J 1997; 2(2): 102~107)

Key Words: *Melanocyte, Cl^- channel, Voltage-dependence*

INTRODUCTION

Ion channels are pore-forming membrane-spanning proteins that mediate passive transport of ions across the cell membrane and intracellular organelles. Since the patch-clamp technique allows recording of ionic currents in relatively small cells such as secreting cells and immune cells, efforts to identify ion channels in the skin cells were made. Mauro and colleagues (1995)[1] have demonstrated that Ca^{2+}-permeable nonselective cation channels are expressed in epidermal keratinocytes and blocking these channels with amiloride inhibited keratinocyte proliferation. The presence of TTX-sensitive Na+ channels and TEA-sensitive and TEA-insensitive K+ channels have also been identified in foreskin- derived cultured human melanocytes[2] and melanoma cell line IRG1[3,4]. Nilius and Wohlrab (1992)[4] have further shown that isoproterenol decreased open probability of the TEA- insensitive A-type K+ channel in melanoma cells, indicating that this channel is regulated by cAMP. Both TEA and cAMP which inhibited activity of this channel also reduced 3H-thymidine incorporation, suggesting that K+ channels regulate cell growth and proliferation[4].

Interest on Cl^- channels has recently increased because of their functional importance; Cl^- channels have an inhibitory effect on synaptic transmission in neurons[5], control resting membrane potential in muscle cells[6,7], and involved in secretion in epithelial cells[8], and regulate cell volume and intracellular pH in various types of cells[9,10]. Although Cl^- channels of the cell membrane are involved in various physiological functions, the presence and function of this channel in melanocytes of the skin have not been studied. The present study is aimed to identify Cl^- channels in cultured human melanocytes and we report for the first time the expression of voltage-dependent Cl^- channels in human melanocytes.

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MATERIALS AND METHODS

Chemicals

Isobutylxanthine (IBMX), cholera toxin, phorbol 12-

myristate 13-acetate (PMA), and ethylene glycol-bis(β-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), N-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), N-methyl-D-glucamine (NMDG) and CsCl were purchased from Sigma Chemical Co. (St. Louis, MO), Ham's F-10, heat-inactivated fetal calf serum and penicillin/streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD), and bovine pituitary extract (BPE) was purchased from Clonetics.

Cell culture

Normal human melanocytes were isolated from neonatal foreskins as previously described[11]. Briefly, subcutaneous fat and blood clot were removed from neonatal foreskin. The foreskin specimen was cut into pieces and incubated in 0.25% trypsin solution in phosphate-buffered saline for 12 hr at 4°C. The epidermis was separated from the dermis, and melanocytes are released into the medium by gentle agitation. The resulting single melanocytes were plated into 25 ml culture flasks. The cells were maintained in Ham's F-10 medium supplemented with 13 μg/ml BPE, 250 μM IBMX, 10 ng/ml cholera toxin, 50 nM PMA, 10% heat-inactivated fetal calf serum, 1% penicillin/ streptomycin with 5% CO_2 at 37°C. For patch-clamp experiments, melanocytes were plated onto 35 mm plastic Petri dishes (Nunclon, Denmark) and used within 2 days after plating. Cells from the second and third passages were usually used for the experiments. Cell viability was tested by trypan blue exclusion and was greater than 95%.

Solutions

The bathing medium was NMDGCl saline consisting of (in mM); 140 NMDGCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, adjusted to pH 7.4 with NMDG. The standard pipette solution contained 135 CsCl, 10 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES adjusted to pH 7.4 with CsOH. Intracellular Ca^{2+} was buffered to a low concentration (10 nM) to prevent possible activation of any Ca^{2+}-dependent currents. Free Ca^{2+} concentrations in these pipette solutions were calculated using a computer program written by Dr. J. Kleinschmidt (New York University, NY). Osmolalities of the bath and pipette solutions were 290-295 and 280-285 mosmol/kg H2O, respectively, measured with a freezing point depression osmometer (Osmette A, Precision Inc., Natick, MA).

Electrophysiological recordings

All recordings were made in the whole-cell patch-clamp configuration using an EPC-9 patch-clamp amplifier (HEKA elektronik, GmbH, Lambrecht, Germany). HEKA Interface hardware and Pulse and Pulsefit (ver. 7.6, HEKA Elektronik) software programs were used to control voltage and to acquire and analyze data. Pipettes were fabricated of borosilicate glass capillary tubing (A-M Systems, Everette, WA) with resistances of about 4~6 M Ω with the pipette solutions used. The series resistance was approximately 15 MΩ during whole-cell recordings, and 70% of the series resistance was compensated. Hence the voltage error was less than 5 mV for the current amplitude of 1 nA. With the pipette in the bath, the zero-current potential was measured, then remeasured after a seal was formed. This new junction potential was then subtracted using the EPC-9 internal circuit during whole-cell recordings. Recordings of whole-cell Cl^- currents began about 10 min after the onset of the whole-cell configuration unless otherwise stated. This allowed diffusion of the pipette contents into the cell and stabilization of the shift in voltage dependence of the Cl^- currents which occurs in the first few minutes[12]. Currents were acquired at 5 kHz and filtered at 1 kHz with the 8-pole Bessel filter in the EPC-9 amplifier. All recordings were made at room temperature (22-24°C). Results are presented as mean ± S.E.M. (n, number of cells).

RESULTS

Fig. 1 shows the time course of whole-cell Cl^- current with high symmetrical Cl^- solutions. We inhibited outward K+ current by replacing K+ with Cs+ and any inward cation current by using NMDG, a bulky cation. With this solution, a test potential of -130 mV from a holding potential of 0 mV elicited inward current. The inward Cl^- current activated slowly and needed approximately 500 msec of hyperpolarization to reach full activation (Fig. 1, upper). The peak current amplitudes were plotted after establishing the whole-cell configuration (Fig. 1, lower). Equilibration of intracellular compart-

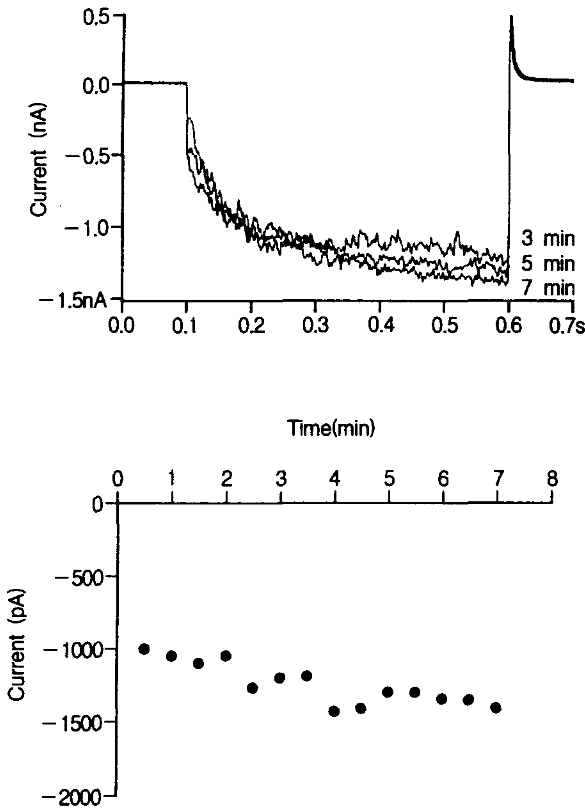

Fig. 1. Time course of Cl^- currents.
The bath contained NMDGCl saline (in mM; 140 NMDGCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH7.4) and the pipette contained CsCl solution (135 CsCl, 10 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH 7.4).
Upper: Cl^- currents were evoked from a holding potential (Vh) of 0 mV at Vc -130 mV applied every 30 sec after the whole-cell recording was established at time=0 min. Traces show the Cl^- currents recorded at 3, 5 and 7 min.
Lower: Peak current amplitudes were plotted as a function of time after establishing whole-cell configuration.

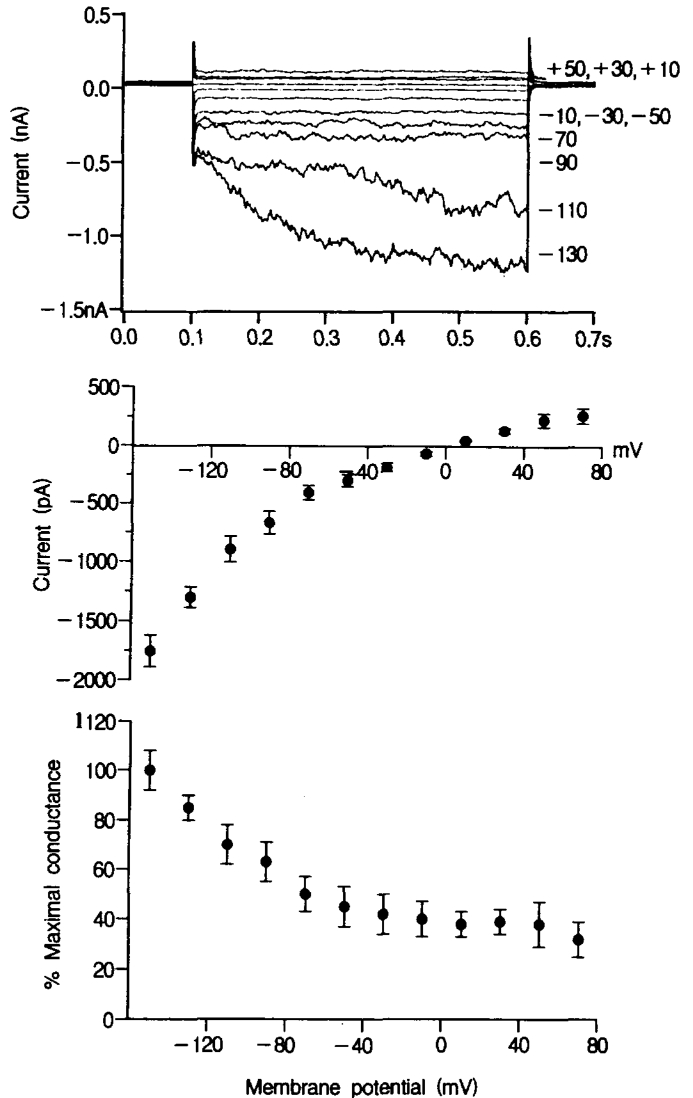

Fig. 2. Current vs voltage relation for activation of voltage-dependent Cl^- current.
The bath contained NMDGCl saline and the pipette contained CsCl saline (see Fig. 2).
Top: Whole-cell Cl^- currents were recorded during 500 msec-long pulses from a holding potential (Vh) 0 mV to voltage steps (Vc) between 50 mV and -130 mV applied every 60 sec in 20 mV increments. Currents were measured 10 min after establishing whole-cell configuration.
Middle: Current vs. voltage (I-V) relation for whole-cell Cl^- current. Average amplitudes between 450 and 500 msec were plotted as a function of voltage. The data points indicate mean ± S.E.M. (n=10).
Bottom: Conductance vs. voltage (g-V) relation for steady-state Cl^- current was determined as a function of voltage. The average amplitudes at each voltage step (Vc) was normalized to the maximum Cl^- conductance obtained at Vc -130 mV. The data points indicate mean ± S.E.M. (n=10).

ments was achieved at 4 min and the whole-cell Cl^- current remained stable for about 30 min. During this period, we have not observed rundown of the current and any changes in kinetic parameters.

Current vs voltage relation was obtained with the standard pipette solution (Fig. 2). At potentials in the range of 50 mV to -10 mV, channels were rarely open. For voltages more negative than −30 mV, inward current began to activate (Fig. 2, top). Maximum current amplitudes varied considerably depending on the size of dendrites. Approximately 15-20 μm-long bipolar or tripolar melanocytes were taken for recordings, and the mean peak current at −130 mV was −1300 ±86 pA (S.E.M., n=10). When maximum current were plotted as

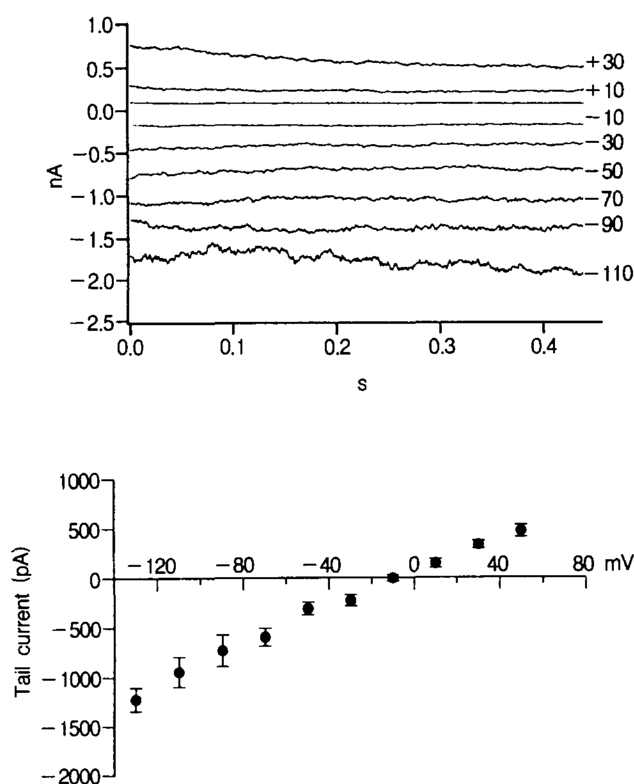

Fig. 3. Tail currents and reversal potentials in whole-cell recordings.
Upper: Tail current beginning 10 min after establishing a whole-cell recording. Vh 0 mV followed by 1 sec-long prepulse to -130 m Vc was given (traces not shown). Tail currents were measured 2-3 msec after each voltage step (Vc), i.e. after the capacitative current has settled. Tail currents between 50 mV and -130 mV are shown.
Lower: Current vs. voltage relations for tail currents. Tail currents measured as above (upper) were plotted as a function of voltage. Values are mean ± S.E.M. (n=6).

a function of the test potential, the current-voltage relationship showed inward rectification (Fig. 2, middle). From the conductance vs. voltage relation, the half-maximal voltage for activation was −60 mV (Fig. 2, bottom).

We measured the reversal potential (Erev) of this inward current by examining the tail currents (Fig. 3). A hyperpolarizing prepulse (−130 mV) was given to activate Cl^- channels from a holding potential of 0 mV, which was then followed by stepping to test potentials from 50 mV to −130 mV (Fig. 3, upper). Tail current amplitudes were measured immediately after the capacitative current had settled. At voltages more positive than Erev the tail currents were outward and the channels showed slow voltage-dependent inactivation. At less negative potentials the tail currents were inward and rarely inactivated. Instantaneous tail currents were plotted as a function of test voltages in Fig. 3 (lower). The tail currents were linear at entire voltage ranges. The reversal potential seen as the zero-current level was approximately -10 mV, which is close to ECl = 0 mV in this condition, suggesting that human melanocytes express highly selective Cl^- current.

DISCUSSION

In this study we demonstrated that human melanocytes express voltage-dependent Cl^- currents. This current was time-dependent and was activated by more hyperpolarizing voltages. Several types of chloride channels have been identified on the basis of single channel conductance, kinetics, voltage dependence, gating, anion selectivity, pharmacology, and biochemical regulation[7]. On the basis of these criteria, slowly activating hyperpolarization-activated Cl^- currents have been observed in oocytes[13], Leydig cells of rat testis[14], granular duct cells of mouse mandibular glands[15], human intestinal T84 epithelial cells[16] and oocytes expressing ClC-2 proteins[17]. Thiemann and colleagues (1992)[17] have further demonstrated broad expression of this Cl^- current in rat heart, brain, lung and liver and fibroblastic, neuronal, and epithelial cell lines. Here we report that this current is also expressed in human melanocytes.

Pharmacology of hyperpolarization-activated Cl^- currents has been studied. Whereas this current was reported to be sensitive to DPC, NPPB, and Cd^{2+}, but insensitive to DIDS and Ba^{2+} in T84 cells[16], Noulin and Joffre (1993)[14] reported that the current was sensitive to DIDS, A9C, and DPC in Leydig cells. The degree of inhibition by these blockers will help further characterization of the Cl^- current in human melanocytes. The hyperpolarization-activated Cl^- current observed in melanocytes is distinct from cAMP-activated outwardly-rectifying Cl^- currents, since cAMP did not affect amplitudes and kinetics the hyperpolarization-activated Cl^- current (data not shown). This current also does not appear to require Ca^{2+} for its activity. An increase in intracellular Ca^{2+} to 0.5 μM with 1 mM EGTA did not increase the current amplitude (I. Chung, unpublished data). This channel does not appear to require phosphorylation for activation,

since intracellular diffusion of MgATP did not change the current amplitudes and kinetics (I. Chung, unpublished data). Further studies are required to find out regulating molecules of this channel.

Hyperpolarization-activated Cl^- currents were further characterized by their threshold potentials. Threshold voltages of the currents were −40 mV in T84 epithelial cells[16] and −100 mV in the oocytes expressing ClC-2 proteins[17]. Because of the highly hyperpolarizing threshold voltage, Thiemann et al. (1992)[17] suggested that this channel might have a housekeeping role in these cells. Nevertheless, exposure of the oocytes to hypotonic solutions activated the ClC-2-related Cl^- current within a physiological voltage range, indicating that ClC-2 channels may regulate the cell volume[7]. Our results showed that half-maximal activation of the Cl^- current was observed at -60 mV, which is within the physiological voltage range, indicating that this channel may serve physiological functions in melanocytes. Our working hypothesis is that the hyperoplarization-activated Cl^- channel may control the intracellular Cl^- concentration by preventing an excessive increase in Cl^- concentration under the basal condition or after agonist stimulation. The second hypothesis is that activation of hyperpolarization-activated Cl^- current may regulate intracellular Ca^{2+}. In mast cells and secreting cells opening of outwardly rectifying chloride channels clamped the membrane voltage at negative values, and thereby increased the driving force for entry of external Ca^{2+} [18]. Hence, activation of the hyperpolarization-activated Cl^- current may help to clamp membrane potential of melanocytes at more depolarizing potentials, preventing an overload of intracellular Ca^{2+}. The functional importance of this channel requires further investigation.

Melanocyte is a dendritic cell located at the basal layer of the skin, from which it differentiates towards the upper epidermal layers. It possesses highly organized spherical membranous organelles, termed melanosomes. Melanosomes synthesize melanin inside their membrane by tyrosinase. The resulting pigmented melanosomes are eventually transferred to the surrounding keratinocytes along dendrites[19]. Presently it is known that melanin production and melanocyte proliferation are affected by many factors such as UV-irradiation, cytokines, growth factors secreted from keratinocytes and fibroblasts[19], and Ca^{2+} [20]. Development of new treatments for pigmentary disorders will be possible after deep understanding of melanocyte physiology and melanogenesis. For the past several years embryological studies have revealed the neuro-ectodermal origin of human melanocytes. Also biochemistry and histochemistry in fixed and stained skin have made a great progress toward understanding melanogenesis. Ion channels, one of the most abundant membranous proteins in neurons, serve various physiological functions such as regulation of membrane excitability, secretion, outgrowth, and movement. The patch-clamp technique which allows recording of proteins' conformational changes within a microsecond range helps to understand dynamic aspects of various cellular events. Here, we propose an approach to understand melanogenesis and physiology of melanocytes which have neuroectodermal nature.

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