Endotoxin-Induced L-Arginine Pathway Produces Nitric Oxide and Modulates the Ca^{2+}-Activated K^+ Channel in Cultured Human Dermal Papilla Cells

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Endotoxin induces an enzyme that synthesizes nitric oxide (NO) from L-arginine (NO synthase) in vascular smooth muscle cells, macrophages, and fibroblasts, leading to the release of NO. We evaluated the release of NO and its intracellular action on the Ca^{2+}-activated K^+ channel (K_{Ca} channel) in cultured human dermal papilla cells by use of the electron paramagnetic response (EPR) spin trapping method and the patch clamp technique. In dermal papilla cells pretreated for 24 h with endotoxin (1 μg/ml), application of 1 mM L-arginine generated NO, although no measurable release of NO was observed in cells without endotoxin pretreatment, as determined by the EPR spin trapping method. With the patch clamp technique, we found that the K_{Ca} channel of dermal papilla cells had high conductance and was voltage dependent. In addition, after endotoxin pretreatment, the extracellular application of 100 μM L-arginine modulated the K_{Ca} channel in the cell-attached patch configurations. In inside-out patch configuration, however, NO produced by L-arginine itself did not modulate the K_{Ca} channel. This modulation of the K_{Ca} channel was suppressed by pretreatment with 100 μM N^{ω}-nitro-L-arginine methyl ester, an inhibitor of inducible and constitutive NO synthases. Methylene blue, a blocker of guanylate cyclase, inhibited the L-arginine–induced activation of the K_{Ca} channel. These results indicate that the endotoxin-induced L-arginine pathway generates NO, which consequently modulates the K_{Ca} channel in cultured human dermal papilla cells by increasing of cyclic GMP–dependent phosphorylation. *J Invest Dermatol 106:342–345, 1996*

Nitric oxide (NO) accounts for the biological properties of endothelium-derived relaxing factor (Ignarro *et al*, 1987; Palmer *et al*, 1987) and is an important endogenous mediator in several tissues. Endotoxins and cytokines induce an enzyme that synthesizes NO from L-arginine (NO synthase) in vascular smooth muscle cells (Fleming *et al*, 1990) and macrophages (Di Rosa *et al*, 1990). It recently became apparent that cytokine-treated murine skin fibroblasts release NO (Werner-Felmayer *et al*, 1990). Because the origin of dermal papilla cells, which play an important role in the induction of hair and in hair growth (Arase *et al*, 1990; Limat *et al*, 1993), resembles that of fibroblasts (Messenger *et al*, 1986), we wondered whether the dermal papilla cells release NO.

We also investigated the physiological effect of the NO produced on the potassium channels of dermal papilla cells. The Ca^{2+}-activated K^+ (K_{Ca}) channels are important in the functioning of cardiac muscle cells (Noma, 1983), pancreatic β cells (Cook and Hales, 1984), and vascular smooth muscle cells (Nelson, 1990). Few studies, however, have examined the K^+ channels in hair follicular cells. In addition, NO modulated the K_{Ca} channel in vascular smooth muscle cells (Williams *et al*, 1988; Fujino *et al*, 1991). We therefore tried to characterize the K_{Ca} channels in cultured human dermal papilla cells and to investigate via the patch clamp technique the effects of NO on this channel.

MATERIALS AND METHODS

Cell Preparation Human dermal papilla cells were cultured as described previously by Messenger (1984). Dermal papillae were enucleated from excised hair follicles with an intact bulbous portion **(Fig 1*A,B*)** and then cultured in Eagle's minimum essential medium supplemented with 15% fetal bovine serum. Dermal papilla cells were subcultured after they had grown out from the papillae **(Fig 1*C*)** and achieved subconfluence. Cells from the second to the fifth passage on 9-mm cell culture inserts were used for the electron paramagnetic response (EPR) and those on thin cover slips for the patch clamp experiments. When dermal papilla cells were to be examined by the EPR and the patch clamp, they were pretreated for 24 h with 1 μg/ml endotoxin.

Solutions and Chemicals Ca^{2+}-Mg^{2+}–free phosphate-buffered saline contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4. A high-K^+ solution consisted of 140 mM KCl and 10 mM K-MOPS buffer (pH 7.2). Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)-Ca^{2+} buffer was used to adjust the concentration of Ca^{2+} to less than 5 μM. Free Ca^{2+} concentrations were determined with a K_d of 87 nM. Diethyldithiocarbamate (DETC) was obtained from Aldrich (Milwaukee, WI), and L-arginine was obtained from Gibco Laboratories (Grand Island, NY). Fluorescein isothiocyanate-lipopolysaccharide from Salmonella typhi (endotoxin), N^{ω}-monomethyl-L-arginine, N^{ω}-nitro-L-arginine methyl ester (L-NAME), dibutyryl cyclic GMP,

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Abbreviations: EPR, electron paramagnetic response; K_{Ca} channel, Ca^{2+}-activated K^+ channel; NO, nitric oxide; SNP, sodium nitroprusside.

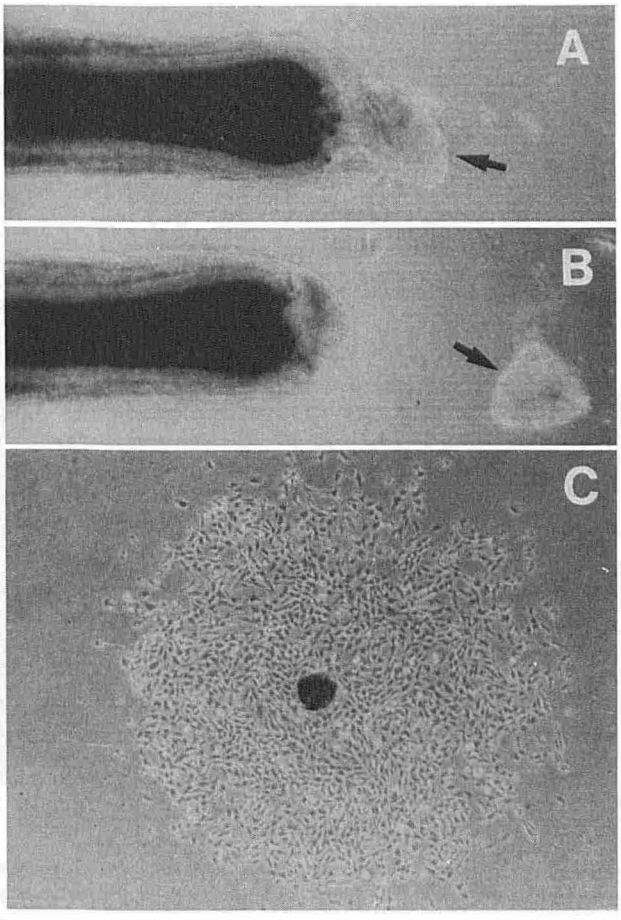

Figure 1. Culture of human dermal papilla cells. The hair bulb was excised, and the dermal papilla was separated from the bulb epithelium (*A,B*). The papilla explants were allowed to proliferate dermal papilla cells (*C*). →, dermal papilla.

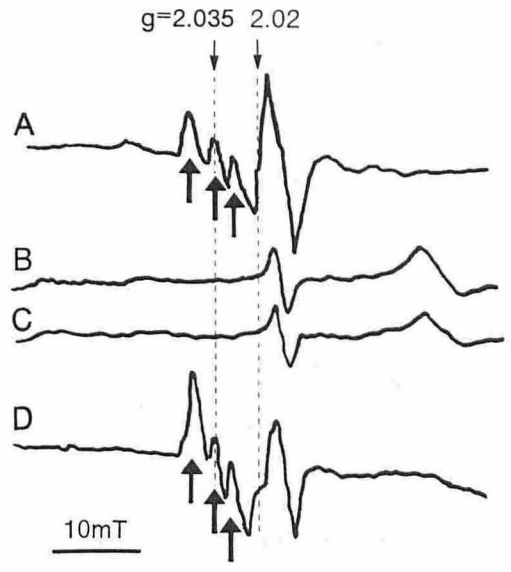

Figure 2. Cultured dermal papilla cells can produce NO. In the EPR spin trapping method, 1 mM diethyldithiocarbamate (DETC) and 1 mM Fe citrate form stable $Fe^{2+}(DETC)_2NO$ complex can be detected as a specific triplet structure. *A*) 1 mM L-arginine was added to dermal papilla cells pretreated with endotoxin. The spectrum was the same triplet structure as the control as the arrows indicate. *B*) No endotoxin pretreatment. *C*) Cell free. No signal from the NO radicals was observed in either Charts *B* or *C*. *D*) Positive control of NO production by 100 μM SNP.

and methylene blue were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium pentacyanonitrosylferrate (III) dihydrate (sodium nitroprusside [SNP]) and tetraethylammonium ion were obtained from Wako (Osaka, Japan).

Measurement of NO Production Production of NO was measured by the EPR spin trapping method developed by Vanin *et al* (Mülsch *et al*, 1992). In this method, DETC and Fe form a complex to trap NO, and stable NO-Fe-DETC complex can be detected by EPR. Cultured human dermal papilla cells were incubated for 24 h in growth medium with or without 1 μg/ml endotoxin. Before the experiment, cells were washed with Ca^{2+}-Mg^{2+}–free phosphate-buffered saline solution. Then 1 mM L-arginine, 1 mM Fe-citrate, and 1 mM DETC were added to 0.6 ml of Ca^{2+}-Mg^{2+}–free phosphate-buffered saline solution with dermal papilla cells. After incubation for 2 min, the supernatants of samples were put into 4-mm diameter EPR tubes (Wilmad Glass, Buena, NJ), and were immediately frozen by immersion in liquid nitrogen until measured. EPR spectra were measured with a JEOL FE-1XG spectrometer (Nihon Denshi, Tokyo, Japan) at liquid nitrogen temperature (−196°C). The measurement conditions were as follows: microwave frequency, 9 GHz; microwave power, 20 mW; modulation amplitude, 0.63 mT at 100 kHz. For positive control we treated 100 μM nitroprusside with Fe and DETC.

Electrophysiological Measurements Membrane currents were recorded in the cell-attached and inside-out configurations with a patch clamp amplifier (Model EPC-7; List Medical Electronics, Darmstadt, Germany), as described by Hamill *et al* (1981). Soft-glass patch pipettes prepared with an electrode puller (PP-83; Narishige Scientific Institute Laboratory, Tokyo, Japan) were coated with Sylgard before use. The electrical resistance of the patch pipettes was 5 to 7 MΩ for single-channel recording. In the cell-attached configuration, the bath solution contained 140 mM KCl, 10 mM K-MOPS, and 10 μM Ca^{2+}, and the pipette solution contained 140 mM KCl, 10 mM K-MOPS, 1 μM Ca^{2+}. In the inside-out patch configuration, the bath solution was 140 mM KCl, 10 mM K-MOPS, and 3×10^{-8} M Ca^{2+}, and the pipette solution was 140 mM KCl, 10 mM K-MOPS, and 1 mM Ca^{2+}. The membrane potential (V_m) was +20 mV. Experiments were performed at 35–37°C. Data were stored with a PCM recorder (Model PCM-501ES; Sony, Tokyo, Japan) with a low-pass filter (3 kHz), and single-channel currents were analyzed with Axograph (Axon Instruments, Foster City, CA). Channel open probability (Np_o) was determined from current amplitude histograms and the equation:

$$Np_o = \sum_{n=1}^{N} (n \cdot Pn)$$

where N is the number of channels in the patch, n is the number of channels in the open state in a patch, and Pn is the area of peak in the histogram.

Results are expressed as means ± SEM. Statistical analysis utilized the Wilcoxon test. A p value of <0.05 was considered statistically significant.

RESULTS

Cultured Dermal Papilla Cells Can Produce NO To determine the production of NO from cultured human dermal papilla cells qualitatively, we compared the EPR spectrum of each sample with that of SNP as a source of NO. In this method, DETC and Fe form a complex to trap NO, and the relatively stable $Fe^{2+}(DETC)_2NO$ complex can then be detected as a specific triplet structure. When L-arginine, DETC, and Fe were added to dermal papilla cells pretreated with 1 μg/ml endotoxin, a triplet signals centered around g = 2.035 and 2.02 appeared. Similar results were obtained in the other three experiments **(Fig 2*A*)**. Without endotoxin pretreatment **(Fig 2*B*)** or cell free **(Fig 2*C*)**, no signal from the NO radical was observed, as indicated by the absence of triplet signals. As shown in **Fig 2*D***, EPR spectra of $Fe^{2+}(DETC)_2NO$ showed triplet signals induced by SNP. The pattern of triplet signals induced by endotoxin and L-arginine was the same as that induced by SNP. These results suggest that cultured dermal papilla cells can produce NO. In the presence of 1 mM N^{ω}-monomethyl-L-arginine, a specific blocker of L-arginine–NO pathway (Moncada *et al*,

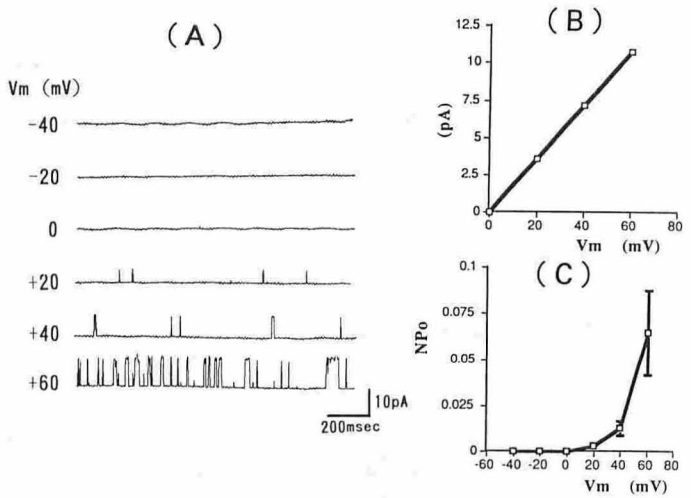

Figure 3. Cultured dermal papilla cells have the K_{Ca} channel. *A*) K_{Ca} channel currents in cell-attached patches of cultured human dermal papilla cells at various membrane potentials (V_m). This channel had a large amplitude and was observed only at depolarized membrane potential. The bath solution contained 140 mM KCl, 10 mM K-MOPS, and 10 μM Ca^{2+}; the pipette solution contained 140 mM KCl, 10 mM K-MOPS, and 1 μM Ca^{2+}. Upward deflections indicate outward-directed transmembrane currents. *B*) The current-voltage relation obtained by plotting the peak values of current amplitude against V_m in the cell-attached patch configuration. The conductance was 179.3 ± 13.1 pS (n = 9). This channel was voltage dependent. *C*) The effect of Vm on Np_o of the K_{Ca} channel in the cell-attached patch configuration. Values *Error bars*, SEM (n = 5). Np_o increased significantly ($p < 0.05$) with the increase in V_m.

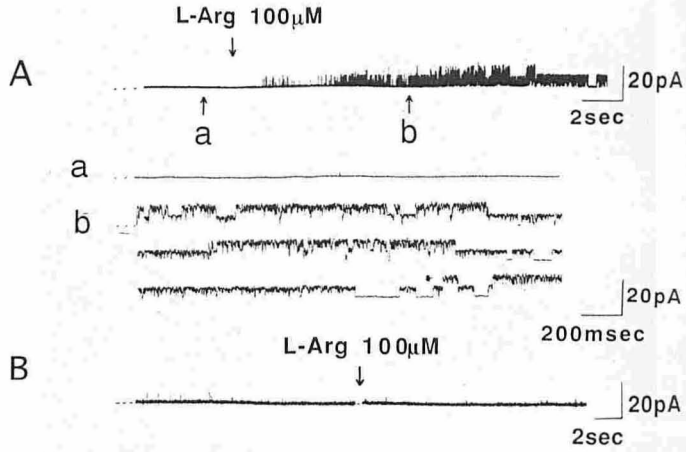

Figure 4. The K_{Ca} channel is modulated by L-arginine (*L-Arg*) in the cell-attached patch configuration with endotoxin pretreatment, but not in the inside-out patch configuration. The membrane potential (V_m) was +20 mV. *A*) The K_{Ca} channel was modulated by extracellular application of 100 μM L-arginine. Upward recordings indicate transmembrane currents in the outward direction, and downward recordings indicate those in the inward direction. *a,b*) Expanded recordings of the indicated portions of *Panel A*. ····, zero current level in this and other figures. The bath and pipette solution were as in **Fig 3**. *B*) In the inside-out patch configurations, the K_{Ca} channel was not modulated by application of 100 mM L-arginine to endotoxin-pretreated cells. The bath solution was 140 mM KCl, 10 mM K-MOPS, and 3×10^{-8} M Ca^{2+}, and the pipette solution was 140 mM KCl, 10 mM K-MOPS, and 1 mM Ca^{2+}. The membrane potential (V_m) was +20 mV.

1991), L-arginine failed to produce NO signals (n = 3; data not shown).

Cultured Dermal Papilla Cells Have the K_{Ca} Channel **Figure 3*A*** shows the channel modulates of cultured human dermal papilla cells in cell-attached patch configurations. This channel had a large amplitude and was observed only at depolarized membrane potential. **Figure 3*B*** shows the current-voltage relations of this channel in the cell-attached patch configurations with a pipette solution containing 150 mM K^+ and with a bath solution of 150 mM K^+. The slope conductance was 179.3 ± 13.1 pS (n = 9). This channel was voltage dependent; that is, the open probability (Np_o) increased with an increase in the membrane potential (V_m), as shown in **Fig 3*C***. In the inside-out patch configurations with symmetrical 150 mM K^+ solutions, the reversal potential of the channel was 0 mV (n = 8). With a pipette solution containing 150 mM K^+ and a bath solution of 50 mM K^+, the reversal potential was +20.0 ± 0.8 mV (n = 8), which was close to the calculated equilibrium potential for K^+ of +29 mV, suggesting that this channel is highly K^+ selective. In the inside-out patch configuration, the K^+ channel was blocked by the application of 10 mM EGTA to the cytosolic face of the membrane, suggesting that this K^+ channel is the K_{Ca} channel (n = 7, data not shown). The K_{Ca} channel was also blocked by the application of 10 mM tetraethylammonium ion, a K^+ channel blocker, to the cytosolic side (n = 5, data not shown).

NO Produced Cannot Modulate K_{Ca} Channel Directly **Figure 4*A*** shows the recordings of the K^+ channel from dermal papilla cells in cell-attached patch configurations. Without endotoxin pretreatment, the K^+ channel was not modulated by the addition of 100 μM L-arginine or of 1 mM L-arginine (n = 4, data not shown). After pre-incubation of dermal papilla cells with endotoxin (1 μg/ml) for 24 h, the application of 100 μM L-arginine to the bath solution quickly modulated the K^+ channel in the cell-attached configuration (Np_o from <0.001 to 0.634 ± 0.246; n = 4; $p < 0.05$) **(Fig 4*A*)**.

Then we investigated whether the NO by itself could modulate the K_{Ca} channel directly. **Figure 4*B*** shows that NO produced from endotoxin-treated cells could not modulate K_{Ca} channels in excised cell-free inside-out patches, suggesting that NO could not modulate K_{Ca} channel directly. We also tested the effect of SNP, a donor of NO, on the K_{Ca} channel in the excised inside-out patches. Addition of up to 100 μM SNP could not modulate this channel either (n = 3; data not shown).

The K_{Ca} Channel Is Not Activated in the Presence of L-NAME We also studied the effects of L-NAME, an antagonist of the L-arginine–NO pathway, on L-arginine–induced K_{Ca} channel activities **(Fig 5*A*)**. In the presence of 100 μM L-NAME, bath application of 100 μM L-arginine did not activate the K_{Ca} channel (Np_o from <0.001 to <0.001; n = 4).

The K_{Ca} Channel Is Thought to Modulate by cGMP-Dependent Protein Kinase The NO produced is thought to activate soluble guanylate cyclase and to produce cyclic GMP (cGMP). Therefore, in order to investigate the relation between modulation of the K_{Ca} channel and cGMP, we added 20 μM dibutyryl cGMP, a membrane-permeable cGMP. The application of dibutyryl cGMP modulated the K_{Ca} channel (Np_o from <0.001 to 0.248 ± 0.112; n = 3; $p < 0.05$) **(Fig 5*B*)**. We also studied the effects of L-arginine on the K_{Ca} channel in the presence of methylene blue, an inhibitor of soluble guanylate cyclase. **Figure 5*C*** shows that the presence of 10 μM methylene blue, the K_{Ca} channel was not modulated by addition of 100 μM L-arginine (Np_o from <0.001 to <0.001, n = 4). As cGMP-dependent protein kinase plays a control role in vasorelaxation (Robertson, 1993), we tested direct effect of cGMP in the K_{Ca} channel (cGMP-PK) to clarify modulation of K_{Ca} channel was due to cGMP-PK. We further found that application of cGMP did not cause discernible activation of the K_{Ca} channel in inside-out configurations (data not shown; n = 4). These results suggest that the K_{Ca} channel of dermal papilla cells is modulated by cGMP-dependent protein kinase.

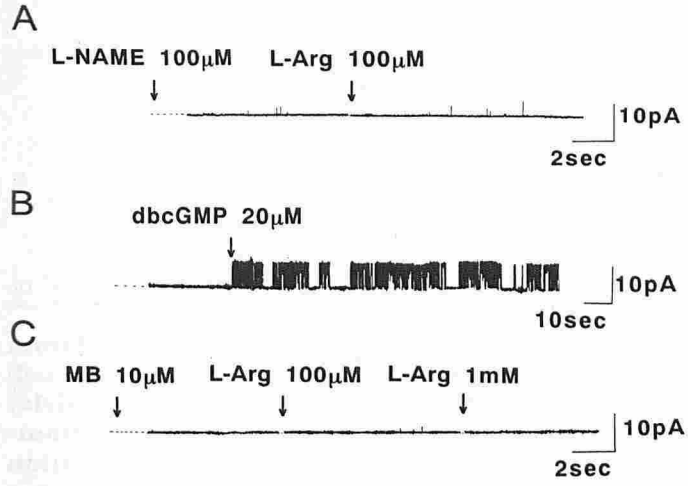

Figure 5. The K_{Ca} channel is not activated in the presence of L-NAME, and the NO produced modulates the K_{Ca} channel by increasing cGMP. *A*) When 100 μM N^{ω}-nitro-L-arginine methyl ester (*L-NAME*) was applied before application of L-arginine (*L-Arg*), the K_{Ca} channel was not activated by extracellular application of 100 μM L-arginine. *B*) The application of 20 μM dibutyryl cGMP (*dbcGMP*) modulated the K_{Ca} channel. *C*) In the presence of 10 μM methylene blue (*MB*), the K_{Ca} channel was not modulated by addition of 100 μM L-Arg and 1 mM L-Arg. Channels were examined in the cell-attached patch modes at a V_m of +20 mV. The pipette and bath solutions were as in **Fig 3**.

DISCUSSION

This study demonstrated that the endotoxin-induced L-arginine pathway produces NO and that the NO produced modulates the K_{Ca} channel in cultured human dermal papilla cells. Studies with methylene blue and dibutyryl cGMP suggest that this activation of the K_{Ca} channel is modulated by cGMP-dependent phosphorylation.

There are at least two types of NO synthase in several tissues. One is a constitutive type that releases NO for short periods in response to various stimulations. The other, an induced type, is induced by endotoxins and cytokines in several tissues, including vascular smooth muscle cells (Fleming *et al*, 1990) and macrophages (Di Rosa *et al*, 1990). Once this enzyme is expressed, NO generation continues for long periods. There is no report about NO synthase in the dermal papilla cells.

Dermal papilla cells play an important role in hair growth and have been shown to produce a factor (or factors) that enhances the growth of follicular epithelial cells (Arase *et al*, 1990; Limat *et al*, 1993). In this study, endotoxin-pretreated dermal papilla cells also produced NO. Recently, it was reported that murine dermal fibroblasts also produce nitrite and nitrate upon treatment with cytokines (Werner-Felmayer *et al*, 1990). In murine keratinocytes, nitric oxide produced by keratinocytes is important in the control of cellular proliferation (Heck *et al*, 1992). Conclusions as to the physiological/functional role of the NO produced in dermal papilla cells could not be determined here, given the scope of the study, and further studies are necessary. We did find, however, that the NO produced modulates the K_{Ca} channels of dermal papilla cells. In other cells, the release of NO was reported to be related to various functions (e.g., nonspecific immunity, relaxation of smooth muscle, and cytotoxity) (Moncada *et al*, 1991). The K_{Ca} channels are ubiquitously distributed in cells and tissues that play an important role in secretion in the endocrine system and in repetitive firing and after hyperpolarization in some neurons and myotubes. The K_{Ca} channels also play an important role in potassium movements in some epithelia (Latorre *et al*, 1989). In vascular smooth muscle cells and in other cells, the K_{Ca} channels are modulated by cGMP (Williams *et al*, 1988; Fujino *et al*, 1991). The results of this investigation showed a similar finding for dermal papilla cells: the intracellular product of NO, cGMP, also modulates the K_{Ca} channel in these cells, in intact cell-attached patches; however, NO itself could not modulate the K_{Ca} channel directly in excised inside-out patches.

The physiological role of NO in modulating the activity of the K_{Ca} channel in dermal papilla cells is unclear. Further studies are needed to explain the relationship between physiological functions and the K_{Ca} channel of dermal papilla cells.

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