

## INVESTIGATIVE REPORT

## Defective Cyclic Guanosine Monophosphate-gated Calcium Channels and the Pathogenesis of Psoriasis

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**A positive association between intake of calcium channel blockers and psoriasis has been observed recently. Intake of blockers of voltage-gated calcium ion channels is associated with outbreaks of psoriasis after a latent period in patients with and without a previous family history of psoriasis. This suggests that interfering with calcium influx may trigger psoriasis. Calcium influx also occurs via cyclic guanosine monophosphate-gated channels; human keratinocytes contain functional and non-functional (splice variants) versions of these channels. We show here that keratinocytes and skin from psoriatic individuals express higher levels of mRNA encoding a non-functional cyclic guanosine monophosphate-gated calcium channel and that high expression of the splice variant by transfection of cells in culture leads to loss of protein expression for the functional cyclic guanosine monophosphate-gated Ca<sup>2+</sup> channels. Key words: calcium; calcium channel; cyclic guanosine monophosphate (cGMP); differentiation; keratinocyte; psoriasis.**

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A recent report of a case-controlled study by Cohen et al. (1), describing an association between intake of calcium channel blockers and psoriasis, suggests that defective calcium channel function may trigger psoriasis. The authors report that intake of calcium channel blockers (nifedipine, felodipine and amlodipine), which block voltage-gated calcium channels, is associated with outbreaks of psoriasis after a latent period (median = 28 months) in patients with and without a previous family history of psoriasis.

An increase in intracellular calcium has been shown to be necessary for the terminal differentiation of normal human keratinocytes and the induction of differentiation markers such as transglutaminase, involucrin, loricrin and profilagrin (reviewed in (2)).

Induction of these proteins plays an important part in cell stratification and in cornification. It is known that keratinocytes in the skin of psoriatic patients undergo incomplete differentiation; finally, there are defects in the calcium gradient between the basal and cornified layers in the epidermis of skin from both lesional and non-lesional skin sites in these patients (references in (2)). Moreover, Karvonen et al. (2) have shown that keratinocyte cultures derived from both lesional and non-lesional psoriatic skin were deficient in their ability to mobilize intracellular calcium stores following application of the calcium-releasing agent thapsigargin. These cells were also unable to activate influx of extracellular calcium (capacitive calcium inflow). Defects were also found in gap-junctional calcium signalling. This failure of the psoriatic-derived cultures to elevate internal calcium levels to the degree seen in normal keratinocytes points to an inherent error in calcium-handling, because the response was maintained in cells that had been kept in culture up to the fifth passage. Several reports show that voltage-sensitive calcium channel blockers fail to block early differentiation events of keratinocytes (3, 4), but do block late differentiation, either by direct blockade of voltage-sensitive calcium channels (5, 6) or by blocking potassium channels (7). For this reason, we decided to look at the expression of other calcium-permeable channels in psoriatic skin.

Five years ago a cyclic guanosine monophosphate (cGMP)-gated ion channel was identified and cloned from normal human keratinocytes by one of our groups (8). This channel has sequence homology to a family of cGMP-gated channels found in other tissues, but the channel from keratinocytes can also exist as splice variant forms. These channels have been shown to be permeable to calcium ions and to be involved in the rise in intracellular calcium (reviewed in references in (8)). We believe that opening this channel in keratinocytes is a significant mechanism for the entry of extracellular calcium in response to signals to raise intracellular calcium. The reasons for this can be summarized as follows: We have shown that this channel exists in four alternatively spliced variants and that increasing the external calcium concentration from 0.07 mM to

1.2 mM induces the expression of the mRNA for the full length transcript (hkcGMPc-1, detectable as a 753 base pairs (bp) RT-PCR product) in normal keratinocytes (8). Another PCR product of 495 bp was constitutively expressed and represents the alternatively spliced hkcGMPc-4 transcript, which has deletion of exons 6, 7 and 8 (8). Thus, expression of the full-length transcript of hkcGMPc-1 is associated with terminal differentiation of human keratinocytes.

In the mouse, a related calcium signalling protein, the CaR, was genetically inactivated by Oda et al. (9) and expression of the full-length transcript was disrupted. Studies with these animals showed that their keratinocytes did not respond to extracellular calcium increases and that the skin of these mice had abnormally low levels of loricrin mRNA and protein expression. In terms of defective expression of differentiation markers, it would seem that the epidermis of these knockout mice has significant similarities to the skin of psoriasis patients (9). This suggests that the expression of all components of the calcium signalling pathway is essential for normal epidermal differentiation and calcium entry in the mouse. Since the spliced variant of the cGMP-gated channel, hkcGMPc-4, is non-functional, we would expect that expression of this variant, and not the complete cGMP-gated channel, could produce defective epidermal differentiation.

## PATIENTS AND METHODS

### *Patients and biopsies*

Patients and biopsies are described in detail elsewhere (10). Briefly, 25 patients (9 females and 14 males) suffering from typical psoriasis vulgaris were enrolled in the study. Their ages ranged from 19 to 74 years, mean  $41.8 \pm 17.5$ . Duration of disease was 0.5 to 27 years, and duration of the last outbreak of psoriasis was assessed as between 2 and 24 weeks. The PASI range in patients studied was 8.0 to 42.3, with a mean value of  $23.8 \pm 10.4$ . None of the patients had received any topical or systemic anti-psoriatic therapy in at least 3 weeks prior to biopsies being taken. Biopsies were obtained from the psoriatic lesions of all patients. Additional biopsies of non-lesional skin (at least 10 cm from the lesions) were taken in 14 of them. Skin from 6 normal individuals served as controls. Informed consent was given by all the persons included in the study, which was carried out as prescribed in the Helsinki Convention. An ethics board at the Medical University of Wroclaw reviewed the application for biopsies used in Fig. 2, and ethical approval was granted by the Lothian Hospitals Trust to biopsy patients attending the psoriasis clinic of the Royal Infirmary of Edinburgh for skin to use for establishing keratinocyte cultures, as described elsewhere (11).

### *Cell cultures*

Keratinocytes were established from non-lesional skin from four individuals with psoriasis and from six normal age-matched controls (11). Cultures were of matched passage number and were grown in keratinocyte media (KGM – Gibco Life Sciences, Paisley, Scotland). The HEK293

human embryonic kidney cell line was maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% foetal calf serum.

### *Reverse transcription polymerase chain reaction (RT-PCR)*

We performed reverse transcription-polymerase chain reaction (RT-PCR) (12) on RNA extracted from keratinocyte cultures established from both normal and non-lesional psoriatic skin and from biopsies from normal and non-lesional and psoriatic skin (10) using semi-quantitative RT-PCR, with [ $^{32}$ P]-labelled primers, which amplify products for both the spliced variant and the full-length channels, which are different sizes (8). We compared the relative expression of mRNA for the spliced variant (hkcGMP-4, 495 base pairs [bp] PCR product (8)) with that of the full-length channel transcript (hkcGMPc-1, 753 bp product) in skin biopsies from psoriasis patients. Briefly, following RT-PCR, the amplified products were resolved on polyacrylamide gels and products visualized by autoradiography, prior to quantitation by densitometry (10). The amount of product was expressed as a ratio of the signal for the particular channel relative to that for a house-keeping gene amplified in the same samples. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used to normalize RT-PCR product densities for the biopsies (10) and  $\beta$ -actin was used for the keratinocytes (12). The Mann-Whitney test was used to test for statistical significance of differences and values of  $p < 0.05$  were considered significant.

### *Immunohistochemistry*

HEK293 cells were transiently transfected with 2  $\mu$ g complete or alternatively spliced cGMP DNA and beta-galactosidase DNA/2  $\mu$ l of TransIT Keratinocyte Transfection Reagent (PanVera Corp., Madison, Wisconsin, USA)/1 ml medium. For dominant negative experiments, the ratio of complete to alternatively spliced cGMP DNA was varied as noted in Fig. 3. After overnight incubation, the cells were washed with phosphate buffered saline (PBS), incubated for 24 h, then fixed with 3% formaldehyde in PBS for 10 min. Full-length and spliced variant cGMP proteins were labelled using a monoclonal antibody (PMc1D1). Photographs were taken on a Leica TCS-SP confocal microscope. Transfection was confirmed by staining for beta-galactosidase. Negative controls omitting the primary antibody demonstrated that there was no non-specific staining.

## RESULTS AND DISCUSSION

Expression of the two forms of the cGMP channel in keratinocytes are shown in Fig. 1. All four keratinocyte strains (cultured from non-lesional psoriatic skin derived from different patients) failed to express the 753 bp full-length functional channel, even after 40 cycles of PCR amplification. However, it was expressed in five of six of the normal keratinocyte cultures (albeit, faintly in two) established from normal adult, age-matched skin (Fig. 1a). This difference might be expected to be even greater in fully-differentiated cultures.

We found that expression of the full-length transcript of the channel hkcGMPc-1 (753 bp) was rarely detectable in the skin of either normal or psoriatic patients, but the non-functional splice variant,

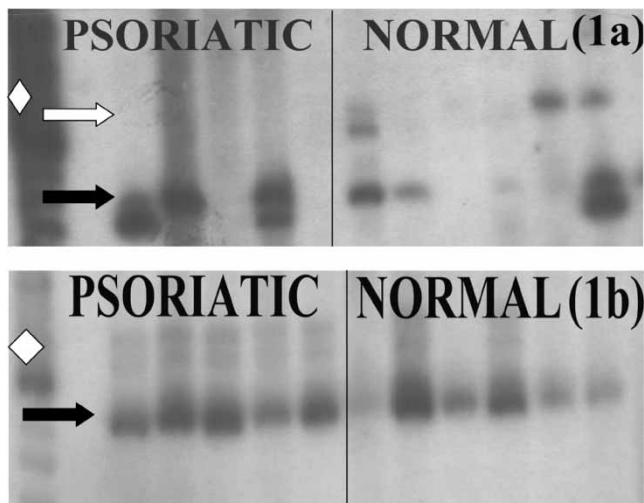


Fig. 1. Visualization by autoradiography of [ $^{32}$ P]-labelled RT-PCR products for cGMP channels (a) and for the housekeeping gene  $\beta$ -actin (b) from non-lesional psoriatic and normal keratinocytes. Each lane represents the amplification of RNA from different donors with cGMP-gated  $\text{Ca}^{2+}$  channel-specific primers, which amplify the full and splice variant channels. Keratinocyte cultures were established from biopsies and RT-PCR performed as in "methods". The 1 Kb molecular weight 100 bp ladder is shown on the left ( $\diamond=800$  bp marker). The solid arrow in (a) indicates the splice variant channel (hkcGMP-4) PCR products (495 bp) and the unfilled arrow points to the position of the full-length channel (hkcGMP-1) products (753 bp). The  $\beta$ -actin product (solid arrow in b) is 661 bp. Curvature of the bands is due to heating of the centre of the resolving gel ("gel smiling").

hkcGMPc-4, was more strongly expressed in lesional psoriatic skin compared with normal skin ( $p=0.0009$ , Fig. 2). The expression in non-lesional psoriatic skin was greater than in normal skin ( $p=0.003$ ), both analysed by Mann-Whitney test. We were unable to amplify channel or G3PDH DNA from 3 non-lesional, 3-lesional and 2 normal biopsies, possibly because of RNA degradation, so the data presented in Fig. 2 are from the pooled data of 2 determinations on normal ( $n=4$ ), non-lesional ( $n=11$ ) and lesional ( $n=22$ ) skin biopsies. The median scores and 25th and 75th percentile values were: normal 0.07, 0.200 and 0.600, respectively; non-lesional 0.400, 0.16 and 0.65; and lesional skin 0.345, 0.25 and 0.71.

The mechanism governing expression of the splice variants is unknown, but the higher expression of the splice variant channel hkcGMPc-4 lesional skin could be due to nucleotide polymorphism in the promoter region of the hkcGMP gene. Moreover, expressing these results as a ratio of the hkcGMPc-4 signal relative to that for the respective G3PDH in the biopsies may actually be an underestimate the increase in expression of the non-functional channel in psoriatic skin compared to normal skin, because of reports that expression of G3PDH and other housekeeping genes is increased in psoriatic skin (13).

We hypothesized that the spliced variant could block

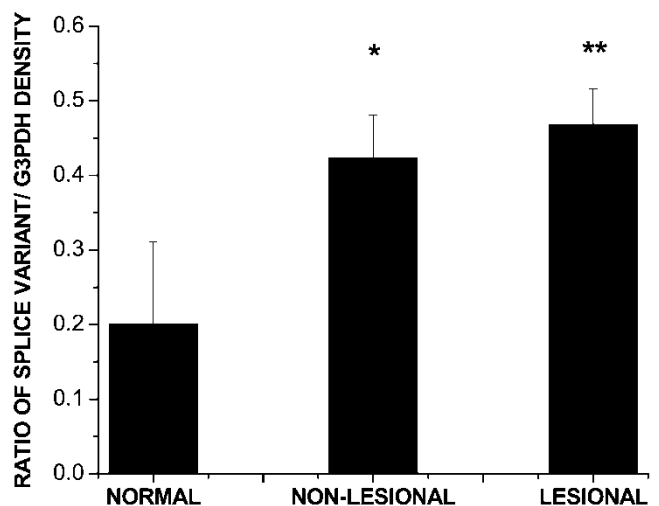
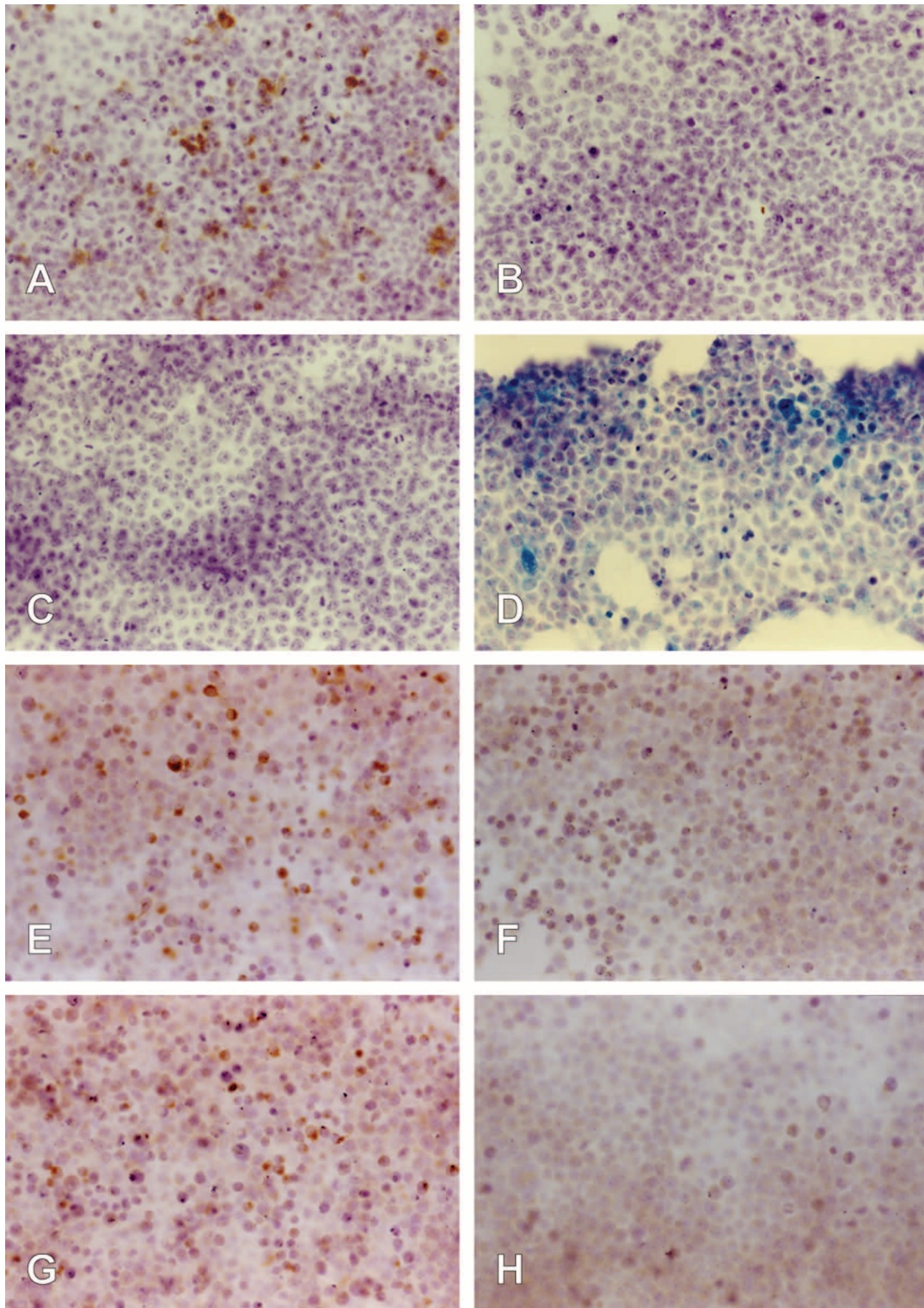


Fig. 2. Higher expression of RNA for the splice variant channel in non-lesional and lesional psoriatic skin compared to expression in normal skin. Biopsies were obtained from normal individuals or from non-lesional and lesional sites of psoriatic patients as described in "methods". Labelled RT-PCR was performed as described in "methods". Autoradiography was performed and the abundance of the PCR products quantitated by densitometry. The ratio of the signal for the splice variant channel product (495 bp) is expressed relative to that for the housekeeping gene G3PDH. These are the means  $\pm$  SEM of results from two experiments. Normal ( $n=4$ ), non-lesional ( $n=11$ ) and lesional ( $n=22$ ). \*Significantly different from normal skin  $p=0.0028$ . \*\*Significant difference from normal skin at  $p=0.0009$  by Mann-Whitney test.

normal channel function through a dominant negative effect. To test whether overexpression of the splice variant blocks expression or assembly of the normal cGMP-gated channels, HEK 293 cells were transfected with both normal and variant channel DNA. These cells normally do not express this channel (Fig. 3). While transfection of full-length cGMP channel DNA resulted in expression of cGMP-gated channels, co-transfection with splice variant and full-length normal cGMP channel DNAs inhibited expression of the normal cGMP-gated channel protein, as detected by immunostaining with the PMcD1 antibody.

Taken together, the results indicate that psoriatic skin expresses more mRNA for the non-functional form of the cGMP-gated calcium channel. Overexpression of the splice variant may inhibit normal calcium influx by blocking expression, through a dominant negative effect, on full-length, functional cGMP-gated channels.

This is consistent with the findings of Karvonen et al. that in established cultures of keratinocytes from both the lesional and non-lesional skin of psoriatic patients there is a subnormal calcium influx (2). The lack of appropriate channels for calcium influx is underlined by the work of Menon & Elias (14), who showed an abnormally high concentration of calcium ions in the suprabasal layer of psoriatic epidermis. Furthermore, it



*Fig. 3.* The spliced variant of the cGMP-gated channel inhibits the expression of the complete cGMP-gated channel. The cDNA of the full-length cGMP-gated channel (A,E,G) and the spliced variant (C,D) was transiently transfected into monolayers of HEK293 cells. The cells were immunostained with the cGMP-gated channel monoclonal antibody PMc1D1. The full-length channel DNA produced a protein detected by the intense brown-orange stain (A,E,G) in the HEK293 cells. No staining was seen when the primary antibody was omitted (B). In contrast, transfection with the spliced cGMP variant DNA did not produce cGMP channel protein (C), although the cDNA was transfected successfully, as shown by (blue) beta-galactosidase staining (D). Expression of the cGMP-gated channel protein was decreased when the spliced cGMP-gated channel variant DNA was co-transfected with the same amount of full-length channel DNA used in A, E and G (2  $\mu$ g) at a ratio of 1:1 (F). Channel expression was abolished when the variant/full-length DNA ratio was increased to 10:1 – the bright-brown immunostaining was lost (H). Magnification =  $\times 250$ .

has been shown that psoriatic keratinocytes produce high levels of nitric oxide (NO) *in vitro* (15) and *in vivo* (16). This would be expected to increase intracellular cGMP – the second messenger for NO – so opening the cGMP-gated channel. In the psoriatic epidermis, keratinocytes fail to differentiate properly, yet they release NO at levels 1000-fold greater than in normal skin. Presumably, this NO causes high levels of cGMP. Despite this, the cGMP-gated calcium channels do not appear to allow differentiation (discussed in (17)). This could be explained by loss of functional channels in the psoriatic skin by high expression of the splice variant (Figs 1–3). In conclusion, these points suggest a defect in calcium channels being fundamental to the inability of psoriatic keratinocytes to differentiate properly. This suggests that the cGMP-gated calcium channel gene may be worth studying as a candidate in the search for genes that predispose individuals to psoriasis.

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