Characterization and Gene Expression of High Conductance Calcium-activated Potassium Channels Displaying Mechanosensitivity in Human Odontoblasts

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Odontoblasts form a layer of cells responsible for the dentin formation and possibly mediate early stages of sensory processing in teeth. Several classes of ion channels have previously been identified in the odontoblast or pulp cell membrane, and it is suspected that these channels assist in these events. This study was carried out to characterize the \( \kappa \)-channels of odontoblasts fully differentiated in vitro using the patch clamp technique and to investigate the HSLO gene expression encoding the \( \alpha \)-subunit of these channels on odontoblasts in vivo. In inside-out patches, \( \kappa \)-channels were identified on the basis of their \( K^+ \)-selectivity, conductance, voltage, and \( Ca^{2+} \)-dependence. In cell-attached patches, these channels were found to be activated by application of a negative pressure as well as an osmotic shock. By reverse transcription-polymerase chain reaction, a probe complementary to \( \kappa \)-calmodulin subunit mRNA was constructed and used for in situ hybridization on human dental pulp samples. Transcripts were expressed in the odontoblast layer. The use of antibodies showed that the \( \kappa \)-channels were preferentially detected at the apical pole of the odontoblasts. These channels could be involved in mineralization processes. Their mechanosensitivity suggests that the fluid displacement within dentinal tubules could be transduced into electrical cell signals.

During tooth development, odontoblasts originating from the neural crest are the cells responsible for the dentin formation (1). These highly polarized cells synthesize the dentin organic matrix made of collagen and noncollagenous macromolecules, which further mineralize. They play a central role in the transport and accumulation of calcium to the mineralization front (2). It is thought that the calcium pathway for dentin formation involves plasma membrane \( Ca^{2+} \)-channels, because specific calcium channel blockers dramatically impair \( Ca^{2+} \)-transport into dentin mineral (3). Besides these \( Ca^{2+} \)-channels, \( Na^+ \), \( K^+ \), and recently \( Cl^- \) channels have also been described in human dental pulp cells cultured in vitro or odontoblasts isolated by enzyme treatment (4–6). It is suggested that these channels play a role in calcium homeostasis as well as in the cellular mechanisms underlying sensory transduction in teeth, a phenomenon that still remains unclear. Indeed, sensory axons have been identified in close contact with the odontoblast bodies or processes corresponding to the proximal end of the cells and running within the mineralized dentin tubules (for review, see Ref. 7). Thus, it is thought that a transductive mechanism for somatic sensation could exist via ion channels in odontoblasts. However, because they form a layer of cells close to the dentin, in situ electrophysiological studies of ion channels in odontoblasts as well as interaction between odontoblastic and neuronal elements proved to be impossible.

We recently developed a unique cell culture system allowing the differentiation of human dental pulp cells into odontoblasts at both the morphological and functional levels (8). We have taken advantage of this cell culture system to use the patch clamp recording techniques to examine, at the single channel level, the characteristics of ion channels in the plasma membrane of odontoblasts. We focus in this paper on high conductance \( Ca^{2+} \)-activated \( K^- \)-channels. Indeed, these channels have been found to be present in the plasma membrane of different cell types and implicated in the regulation of a variety of functions, such as cell firing in neurons, secretion in endocrine or exocrine cells, and myogenic tone in arterial smooth muscle (9–13). Their basic characteristic is that channel opening is induced by an increase in intracellular \( [Ca^{2+}] \) as well as membrane depolarization. The gene encoding the pore-forming \( \alpha \)-subunit of this channel was first cloned in Drosophila (14) and later from various species, including human (HSLO) (15–17). We demonstrate here, in on-cell patches, that these \( K^- \) channels display mechanosensitivity. Furthermore, using RT-PCR, in situ hybridization, and immunohistochemistry techniques, we provide evidence for the expression and distribution of \( K^- \)-channels at strategic locations in odontoblasts in vivo.

**EXPERIMENTAL PROCEDURES**

**Odontoblast Cell Culture**—Dental pulp cells were obtained from sound human third molar germs (14–16 years old) that were extracted for orthodontic reasons. Informed consent was obtained from the patients. Explants were grown in Eagle’s basal medium supplemented with ascorbic acid, antibiotics, 15% fetal calf serum, and 10 mM sodium \( \beta \)-glycerophosphate as described previously (8). After 2–3 weeks of culture, cells exhibiting an eccentric position of the nucleus and displaying an elongated process were used either for electrophysiological recordings or harvested for isolation of total RNA. Fig. 1 shows the typical aspect of these cells under the inverted microscope.

**Odontoblast Cell RNA Extraction and RT-PCR Analysis**—Total RNA were extracted from the cultured cells using the RNeasy kit and protocol (Qiagen, Chatsworth, CA). Purified RNA (3 \( \mu \)g) were reverse-transcribed using random hexamers as primers and converted into cDNA by means of the StrataScript RT-PCR kit (Stratagene, La Jolla, CA). PCR

1 The abbreviations used are: \( K^- \), \( Ca^{2+} \)-activated \( K^- \); RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; Pa, Pascal(s).

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amplification was then realized from a tenth of the RT mixture in 50 μl containing 10 mM tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 units of Taq DNA polymerase (Roche Molecular Biochemicals) and 30 pmol of each Maxi-KCa (HSLO) primer (forward primer: 5'-CAGATTGTGGGATTGTG-3', reverse primer: 5'-CATGACCTTTGGTTATTCTTCC-3') corresponding to bp positions 2824–2845 and 3683–3682, respectively, of the human sequence (17). The amplification was carried out for 35 cycles (1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C followed by 10 min at 72 °C). The PCR product was analyzed on a 1% agarose gel by electrophoresis.

In Situ Hybridization—The material consisted of five sound nontreated human third molars extracted for orthodontic reasons from adolescents with their informed consent. Immediately after extraction, the pulp tissue was carefully removed from the dentin walls and embedded in Tissue Tek OTC compound (EMS, Washington, PA). The specimen were then immersed in liquid nitrogen-cooled isopentane and stored frozen at −70 °C. Cryostat sections (10 μm) were collected on 3-aminopropyltriethoxysilane-coated slides, air-dried, and kept frozen (−70 °C) until treatment. Culture samples were prepared as described previously in detail (8). For detection of the HSLO transcripts, in situ hybridization was performed according to Bleicher et al. (18) using a single-stranded DNA probe with a specific activity of about 2.8 × 10⁶ cpm/pmol. The images were processed using Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA).

Immunohistochemistry—Pulp slices, recovered as described above, were rinsed in PBS and incubated in PBS solution supplemented with normal goat serum (1:50, Institut Pasteur, Paris, France) and 0.2% bovine serum albumin for 30 min at room temperature. After washing, the sections were reacted with polyclonal anti-BKCa or anti-α₁C L-type calcium channel antibodies raised in rabbit (Alomone Laboratories, Jerusalem, Israel). Subsequently, the slices were rinsed, incubated (45 min at room temperature) with Cy3 goat anti-rabbit IgG (Interchim, Jerusalem, Israel). Subsequently, the slices were rinsed, incubated (45 min at room temperature) with Cy3 goat anti-rabbit IgG (Interchim, Jerusalem, Israel) and washed extensively, and sealed from the air with glycerol/PBS (1:1) solution. Sections were examined with a Reichert-Jung Polyvar microscope appropriately equipped.

RESULTS

Characterization of K_Ca Channels in Inside-out Patches—Upon patch excision from odontoblasts in vitro (Tyrode solution in the pipette, K⁺-rich solution containing 2.5 mM Ca²⁺ in the bath), one type of channel with a unitary current amplitude of about 6.5 pA was spontaneously active at 0 millivolt. This channel activity was totally and reversibly suppressed on perfusion of the cytoplasmic face of the patch by an internal solution containing EGTA and no added calcium (Fig. 2A).
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2B shows the conductance properties of this channel. Segments of current traces indicated that unitary current amplitudes were 2.7, 6.2, and 9 pA at −20, 0, and +20 millivolts, respectively, in the presence of 5 mM K+ in the pipette and 4, −4.1, and −9.7 pA at +20, −20, and −50 millivolts, respectively, in the presence of 140 mM K+ in the pipette (Fig. 2B). Additionally, it can be seen that channel opening increased with the depolarization amplitude; in the patch illustrated in the right panel of Fig. 2B, NPo was 0.2, 0.65, and 0.87 at −50, −20, and +20 millivolts, respectively. In the presence of 5 mM K+ in the pipette, the current-voltage relationship displayed an outward rectification and indicated a conductance of 76 picosiemens at 0 millivolt (Fig. 2C). In the presence of 140 mM K+ the currents reversed at 0 millivolt, which is the value of the K+ equilibrium potential under these ionic conditions; the relationship was linear, and the best fit to the mean data indicated a conductance of 200 picosiemens (Fig. 2C). On the basis of their conductance properties, and the dependence of their activity upon intracellular calcium and voltage, these channels were identified as high conductance Ca2+-activated K+ (KCa) channels.

Mechanosensitivity of KCa Channels in Cell-attached Patches—In cell-attached patches established on odontoblasts bathed in a K+-rich solution, brief opening of KCa channels (identified on the basis of their conductance: 100 picosiemens at +60 millivolts) could only be detected at highly depolarized membrane potentials, presumably because of the too low concentration of free Ca2+ inside the cells under these experimental conditions (Fig. 3A). However, we observed that applying negative pressure to the membrane in cell-attached patches gave rise to a reversible increase in channel activity. In the cell-attached patch illustrated in Fig. 3B, a K+ solution containing EGTA and no added Ca2+ was present both in the pipette and in the bath. In the absence of pressure, channel activity increased with membrane depolarization. Applying suction of an amplitude of −4 kPa increased NPo from close to zero to 0.03 at +20 millivolts, from 0.03 to 0.15 at +40 millivolts, and from 0.3 to 1.12 at +60 millivolts, respectively.

In cell-attached patches, at a given membrane potential, KCa channels were found to be activated in a pressure-dependent manner. Fig. 4A illustrates KCa channel activity at different negative pressures in a patch held at +50 millivolts (Tyrode solution in the pipette, K+-rich solution in bath). In this patch, NPo was close to zero in control and increased to 0.4, 0.93, and 1.6 in the presence of −2, −4, and −6 kPa negative pressure amplitude, respectively. The relationship between KCa channel activity and negative pressure level is illustrated in Fig. 4B. The relationship was fitted with a Boltzmann equation (relative NPo = 1/(1 + e[P/Po − Po/k]), where P is the pressure applied to the pipette, Po is the amount of pressure required to induce half-maximal potentiation, and k is the steepness of the relation. The best fit to the mean data was obtained with values of 2.9 kPa for Po and 1.1 kPa for k. Maximum activation occurred at about −6 kPa. In inside-out patches, in the presence of a low calcium concentration (0.7 μM), it was also found that negative pressure gave rise to potentiation of KCa channel activity (data not shown).

Fig. 5 shows that an osmotic shock was also able to induce KCa channel opening in cell-attached patches. In control, cells were bathed in a K+-rich solution containing 300 mM saccharose and no channel opening was detected. Upon superfusion of the cell with a K+-rich solution devoid of saccharose, after a delay of about 10 s, KCa channels began to open and NPo reached a maximum of 0.22. On returning to the hypertonic external solution, NPo progressively decreased and KCa channels completely shut after a delay of 25 s. Similar results were obtained in four other patches.

Detection of HSLO Transcripts by RT-PCR in Cultured Odontoblasts—Analysis of mRNA expression using RT-PCR revealed that the α-subunit was clearly expressed. The products ran at the expected size of 860 bp as shown on Fig. 6. The restriction of the α fragment gave rise to two visible fragments in close agreement with predicted sizes of 537 and 278 bp (lane 3). The 45-bp restriction fragment was too small to be detected. This confirmed that the PCR product analyzed in our studies accurately represented the HSLO sequence encoding the human KCa channel α-subunit.

In Situ Hybridization of HSLO Transcripts in Cultured Odontoblasts and Human Dental Pulp in Vivo—As expected, HSLO transcripts were detected in cultured odontoblasts using in situ hybridization (Fig. 7a). However, we observed a relatively moderate level of transcripts in the cells despite their level of functional expression assessed by electrophysiological recordings.

Experiments conducted on human dental pulp tissue in vivo (Fig. 8) demonstrated that, despite a moderate level of tissue labeling, the highest density was observed in odontoblasts and pulp cells surrounding nerve fibers or arterioles.

Immunocytochemistry of KCa Channels in Cultured Odontoblasts and Human Dental Pulp in Vivo—The Slo protein immunoreactivity was observed in the cultured odontoblasts with
a diffuse dot staining throughout the cells (Fig. 7b). In cryostat sections of extracted pulps (Fig. 9), immunofluorescence of the BKCa channels showed a similar pattern of labeling in the odontoblast bodies, but the immunoreactivity was stronger at the apical pole of the cells. Nerve fibers and some blood vessel walls were also found to be positive. Anti-L-type Ca2\(^{2+}\) channel antibodies gave a positive staining on odontoblasts, which was particularly intense in the apical region of the cells. Control sections showed negligible or no staining in these cells.

**DISCUSSION**

In this study, we report that KCa channels are present in odontoblasts in culture originating from human dental pulp. Our cell culture system allowed us to unambiguously experiment on highly differentiated odontoblasts (8). We showed that odontoblast KCa channels are weakly active in on-cell patches even at highly depolarized membrane potentials but can be activated by applying suction in the pipette or in response to an osmotic shock applied to the cell. KCa channel activity was already described in human pulp cells in culture and dissociated odontoblasts from rat, but questions remain about the identity of the cells under study (4, 6). It was also demonstrated that these channels displayed mechanosensitivity. However, these data were obtained in excised patches in the presence of activating [Ca2\(^{2+}\)] at the cytoplasmic face, and we could not exclude that stretch sensitivity resulted from some alteration of subsarcolemmal components subsequent to patch excision. We showed that stretch activation could be revealed in the absence of calcium at the external face of the membrane (see Fig. 3B). This indicates that the increase in channel activity in response to membrane stretch was not the consequence of an

**FIG. 4.** Effect of application of negative pressure of increasing amplitude on KCa channel activity in cell-attached patches. A, KCa channel currents recorded in the presence of different pressure levels in the pipette (indicated next to each current trace). The membrane potential was held at +20 millivolts. B, relationship between channel activity and negative pressure. Each symbol corresponds to mean ± S.E. (n = 5 patches). The curve was fitted with a Boltzmann equation (see “Results” for details).

**FIG. 5.** Effect of an osmotic shock on KCa channel activity in a cell-attached patch. The patch potential was held at +20 millivolts. In control, an internal K\(^{-}\)–rich solution containing 2.5 mM Ca2\(^{2+}\) and 300 mM saccharose was present in the bath.

**FIG. 6.** Analysis of RT-PCR products of KCa channel α-subunit mRNAs from odontoblasts cultivated in vitro. Lane 1 contains the standard VIII (Roche Molecular Biochemicals). Lane 2 contains PCR product of the α-subunit. The RT-PCR product migrates in the gel to a position in good agreement with its predicted size of 860 bp. Lane 3 shows restriction enzyme products of the α fragment in close agreement with predicted sizes.

**FIG. 7.** a, in situ hybridization with HSLO cDNA in the cultured odontoblasts. Magnification, × 350. b, Slo protein immunostaining in the cultured odontoblasts. Positive dots are clearly detected throughout the cell bodies. Magnification, × 400.
influx of calcium via stretch-activated channels or stretch-induced Ca\textsuperscript{2+} leak. We cannot rule out a possible transduction via intracellular second messenger systems or cytosolic factors. However, the fact that stretch-induced activation was also observed in excised patches favors the hypothesis according to which the membrane stretch directly affects the odontoblast KC\textsubscript{a} channel protein itself or a membrane component closely related to the channel. Activation of KC\textsubscript{a} channels in response to membrane stretch and not mediated by an increase in Ca\textsuperscript{2+} entry has also been observed in osteoblast-like cells, renal cells, smooth and skeletal muscle cells, and neurons (19–23). In these preparations, channel activation was observed in the same range of pressure as in odontoblasts, i.e. half-maximal activation was achieved at pressures around \(-3\) kPa.

In agreement with our electrophysiological data, PCR experiments and in situ hybridization performed in cultured odontoblasts demonstrated expression of the transcripts of the HSLO gene encoding the pore-forming \(\alpha\)-subunit of KC\textsubscript{a} channels. Additionally, the presence of the channel protein was confirmed with specific antibodies. More importantly, using in situ hybridization experiments, we showed that the HSLO gene was expressed by odontoblasts in vivo. Transcripts were also detected in nerve terminals as well as in vascular smooth muscle cells. These latter distributions are not surprising, because KC\textsubscript{a} channels have been shown to be involved in the regulation of smooth muscle tone and excitability of nerve terminals in central and sensory neurons (16, 24). In odontoblasts as well as in nerve terminals and vascular smooth muscle cells, a relatively weak signal was detected. Similar findings were reported by Rosenblatt et al. (25) using in situ hybridization in cochlea, nerve terminals, and vascular smooth muscle cells despite a well established functional expression of these channels in these different cell types. These results could indicate that the KC\textsubscript{a} channel protein undergoes a low turnover.

In vivo immunocytochemical experiments showed that the channel protein was strongly expressed at the apical pole of the.
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Interestingly, the localization correlates to the spatial distribution of \( L \)-type \( Ca^{2+} \) channels. Odontoblasts actively transport calcium to the mineralization front of the dentin matrix. This process is known to take place at the apical pole of the cell and is thought to involve voltage-dependent \( L \)-type \( Ca^{2+} \) channels (3, 26). Recently, it was reported (27) that extracellular \( Ca^{2+} \) increased intracellular free \( Ca^{2+} \) through a mechanism involving both influx of external \( Ca^{2+} \) via \( L \)-type \( Ca^{2+} \) channels as well as release of \( Ca^{2+} \) from internal stores. Colocalization of \( Ca^{2+} \) and \( K_{\text{Ca}} \) channels at the apical pole suggests that \( K_{\text{Ca}} \) channels could exert a negative control of calcium entry by hyperpolarizing odontoblasts and closing voltage-dependent \( Ca^{2+} \) channels in response to an increase in intracellular \( Ca^{2+} \).

The presence of \( K_{\text{Ca}} \) channels displaying mechano-sensitivity in odontoblasts could also have relevance in the sensory transduction phenomenon in teeth. Mechanical stimulation of odontoblasts and fluid flow in dentinal tubules are known to elicit nociceptive responses (28, 29). Additionally, changes in osmotic gradient or probing dentin were shown to increase the discharge rate of pulp cells and evoke action potentials in their primary afferents. Afferent nerve terminals are known to cotound around odontoblasts, and the close association of odontoblast processes and nerve endings (30, 31) has presupposed an interaction between these cells as the earliest step of tooth pain transmission. Stretch-activated \( K_{\text{Ca}} \) channels could be involved in this process. We found that \( K_{\text{Ca}} \) channels open in response to an osmotic shock, which was also recently found to induce an elevation of intracellular \( Ca^{2+} \) in living odontoblasts from sliced dental pulp (32). It can be postulated that, in response to mechanical stimuli, the combination of increased intracellular \( Ca^{2+} \) plus membrane stretch could cause \( K_{\text{Ca}} \) channel opening in odontoblasts. The resulted elevation in the extracellular \([K^+]\) in the restricted cleft delimited by the neuronal and odontoblast membranes may depolarize the nerve endings and lower threshold for nerve firing in the sensory tract. This could explain why \( K^+ \)-containing agents placed into deep dentinal cavities induce a brief burst of high frequency activity in the intradental nerves (33).

In conclusion, the results presented here show that in vitro differentiated human odontoblasts express mechano-sensitive \( K_{\text{Ca}} \) channels. The HSLO transcripts coding for the \( \alpha \)-subunit constituting the pore of these channels are expressed in odontoblasts in vivo. The preferential localization of the Slo protein at the apical pole of the cells in vivo suggests a role of \( K_{\text{Ca}} \) channels in the mineralization process and in the mecha-no-transduction of fluid displacement within dentinal tubules into electrical cell signals.

Acknowledgments—We acknowledge the staff of the “Service de stomatologie de l’hopital St Joseph” Lyon, for collecting tooth samples. We are grateful to Lee Pupe for grammatical review of the manuscript.

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