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Effect of Sustained Mechanical Perturbation on Stretch Sensitivity of G292 Osteoblast-Like Cells

Paul A. G Lingenbrink

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EFFECT OF SUSTAINED MECHANICAL PERTURBATION
ON STRETCH SENSITIVITY OF G292 OSTEOBLAST-LIKE CELLS

Paul A. G. Lingenbrink

D.D.S., University of Washington, 1992

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EFFECT OF SUSTAINED MECHANICAL PERTURBATION
ON STRETCH SENSITIVITY OF G292 OSTEOBLAST-LIKE CELLS

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ABSTRACT

During orthodontic tooth movement, osteoblasts and osteoclasts act in response to forces applied to teeth, which eventually leads to a distortion of the extracellular matrix. The effects of this mechanical stimulation may occur either directly or indirectly, leading to a change in transmembrane potential and increased levels of intracellular metabolites (second messengers). In this study, G292 human osteoblast-like osteosarcoma cells were subjected to varying durations of continuous mechanical perturbation. It was initially determined that stretching cells does not alter cell number. The first experimental aim was to identify a physiological effect in these cells when they were subjected to mechanical stretch. The second aim was to identify mechanosensitive ion channels in G292 cells and characterize their biophysical properties. Ion channels were identified and characterized for their respective channel amplitudes with varying membrane potentials. In addition, conductance, stretch sensitivity, and probability of the channel being open in relation to a specific applied negative pressure were determined.

G292 cells were divided into two groups, stretched cells and controls.
They were then mechanically perturbed, and their electrophysiological properties were evaluated using the patch clamp technique. Overall, 141 total patches were obtained and three ion channels; 20 pS, 80 pS and 200 pS were identified. The focus of the study was on the 200 pS channel due to its ease in recognition and detection. Of these records, only 33, which contained the largest channel could be analyzed (18 stretched and 15 controls). Immediately after stretch application, patches were obtained at 1, 24, and 48 hours. All patches were subjected to a negative pressure ranging from 0 to -10 cm Hg. Results from this study indicate that there was a significant difference between osteoblasts that had been stretched and those that had not. Significant differences were observed between stretched and control cells when determining the probability of a single ion channel being open ($P_{\text{open}}$), and the pressure at which $P_{\text{open}}$ reaches 1/2 of its maximum value. Findings suggest that ion channels in stretched cells were open and responsive approximately three times more than their counterparts in the control cells. Finally, stretched cells reached one half of their maximum ion channel opening at a corresponding pressure of about -3 cm Hg. Control cells obtained this one half maximum value at a pressure of -1.33 cm Hg. It appeared that the control cells quickly reached this maximum value along the
pressure continuum, while the stretched cells took longer to obtain this 1/2 maximum value. Results of this study may help in understanding the ion channel events which may occur in osteoblasts during mechanical perturbation as a model of orthodontic tooth movement.
Cellular events in bone remodeling during tooth movement

Orthodontic tooth movement is a complex process involving application of various forces to malpositioned teeth with the goal of achieving alignment within the dental arches. Initially, the applied force causes a physical displacement of the tooth within the alveolar socket. This movement creates tensions and pressures in the supporting periodontium and surrounding alveolar bone. Subsequently, the pressures and tensions effect the cells of these supporting tissues. Two theories help to explain the transformation of sustained mechanical loads applied to the teeth into the cell reactions that are necessary for bone remodeling (Proffit, 1992). The first theory relates tooth movement to biochemical-responses by the cells and the extracellular components of the periodontal ligament (PDL) and alveolar bone. The second theory refers to tooth movement as a bioelectric phenomena that may occur as a result of mineralized or nonmineralized collagenous matrices. These will be further explained below.

Clinically, the amount of force applied determines the rate and type of
bone remodeling that takes place. When orthodontic stress is applied to the
tooth, and thereby to the PDL and bone, the extracellular fluids of the PDL
shift, and the cells and matrices become distorted. A classic theory of tooth
movement describes the changes in blood flow within the PDL in relation to
applied pressure (Gianelly, 1969). He observed that light forces cause bone
to resorb more rapidly. As the bone resorbed on the pressure side of the
tooth, a relatively slow decrease in the rate of blood perfusion was found,
due to the partial compression between the tooth and alveolar bone within
the periodontal ligament. He also noted that when light forces were applied,
the vascular network of the periodontal ligament remained patent.
Theoretically, this would allow the osteoclast cells to have access to the
surrounding tissues responsible for certain aspects of the remodeling
process. This type of resorption was termed “frontal resorption” and is
efficient, where tooth movement takes place within about seven days (Proffit,

On the other hand, heavy, excessive forces lead to a cellular
compression and a markedly decreased blood flow, where occlusion of the
vasculature is observed. This ultimately leads to a "sterile necrosis" or death
of the surrounding cells compressed between the tooth and alveolar bone
due to a loss in vascularity of this region (Proffit, 1992; Gianelly, 1969; Sandstedt, 1901; Sandstedt, 1904, 1905). This process is also referred to as a "hyalinization" or "cell free" area of the compressed PDL, and tends to appear most commonly in the alveolar crest. The resorption which occurs adjacent to this area of hyalinization is referred to as an area of "undermining resorption", where bone is removed from the medullary side of the alveolar bone instead of from the PDL side (Buck and Church, 1972). These two mechanisms by which bone resorbs, "frontal" and "undermining resorption", are distinct because the latter is much less efficient and takes much longer, typically between 14 and 21 days to be observed clinically (Proffit, 1992).

In a given area, these applied pressures result in a loss of bone mass, called resorption, as opposed to areas of tension, which stimulate apposition of new bone (Grimm, 1972). The association between mechanical forces and bone remodeling was first identified just over one century ago (Wolff, 1892). Bone resorption is dependent on the direction of pressure application, while new bone is deposited in the space that was previously occupied by the tooth prior to movement. Overall, this phenomenon allows the tooth to move through alveolar bone.

Bone remodeling is a dynamic process consisting of bone formation or
deposition, resorption, and maintenance in response to local and systemic biochemical factors. Studies suggest that this process is mediated principally by fibroblasts and osteoblastic cells (Rodan and Martin, 1981; Meikle, Heath and Reynolds, 1986; McSheehy and Chambers, 1986a & 1986b; Heath et al., 1984a). Others have found increases in inter- and intracellular messengers between these cells, which may be elicited in response to bacterial, hormonal, and mechanical stimulation associated with bone remodeling (Heath et al., 1984b; Ngan et al., 1988; Somerman et al., 1990).

Experimental evidence has shown that the open state of ion channels as seen in most cells depends on the stress that is exerted at the cell membrane (Morris, 1990). Others have found that this state may be influenced by the maintenance and composition of the extracellular matrix (Davidovitch, 1991; Ingber and Folkman, 1989). From this it is assumed that when orthodontic forces displace the periodontal ligament, such movement may result in cell perturbation, which alters the flow of Ca^{2+} and other ions in and out of the cell. This in turn has been believed to alter the synthesis of cAMP (Davidovitch, 1991). Others have found that increased levels of cAMP and cGMP were present in alveolar bone and PDL cells following the application of orthodontic forces to teeth (Davidovitch and Shanfeld, 1975).
Another team demonstrated that the synthesis of cAMP coincident with the stretching of cells is dependent on prostaglandin (Somjen et al. 1980). Others have found that the transduction of orthodontic mechanical forces consists in physical deformation of the cell membranes which in turn results in prostaglandin synthesis (Rodan and Martin, 1981). They also found that membrane deformation leads to the activation of membrane-bound adenylate or guanylate cyclases responsible for converting these respective substrates to cAMP and cGMP. Ultimately, these processes may have an effect on the change in membrane tension and could directly lead to differences in the flow of ions in and out of the cell. Eventually, this may all effect the amount and rate of mineralization of osteoid, which is the precursor to bone, secreted by the osteoblast cells. The primary focus of this study will concentrate on osteoblastic cells and their relation to tooth movement as a result of applied mechanical stimulation. This study will go one step further and try to identify the changes in ion channel activity that are present in response to this stimulus.
Characteristics of osteoblasts

Osteoblasts are mononucleated cells that synthesize both collagenous and non-collagenous bone proteins. These proteins are deposited as osteoid, which undergoes mineralization. The osteoblast is thought to be derived either from a multipotent mesenchymal cell or from a perivascular cell (Owen, 1980). Osteoblasts are found lining the bone surface as a cellular layer. These cells undergo two physiologic stages or phases with differing cellular morphology. When osteoblasts are actively synthesizing bone matrix, they have an ovoid appearance. Histologically, they appear to have open-faced nuclei with abundant basophilic cytoplasm (Ten Cate, 1985). When osteoblasts are inactive, they appear flatter or more squamous, with close-faced nuclei. These are termed lining cells. As the synthesized bone matrix released by these cells becomes mineralized, it entraps some of the osteoblasts. Upon full entrapment, these cells are then referred to as osteocytes. The number of osteoblasts that are transformed into osteocytes varies and is dependent upon the rate of bone formation. After entrapment, the osteocytes eventually lose their ability to synthesize and release bone matrix and decrease in volume. Communication between
osteocytes and osteoblasts is maintained by canals within the mineralized bone called canaliculi. This communication between cells is essential for the bone to maintain vitality.

While the osteoblast has its main function of bone formation, a second type of cell, the osteoclast is the cell primarily responsible for bone degradation. An apparent coordination between these two cell types, and the regulation of the number and the activity of these cells has been suggested to be carefully controlled (Rodan and Martin, 1981). Many researchers have described the biological cell events that occur during orthodontic tooth movement (Graber and Swain, 1975; Gianelly and Goldman, 1971; Mostafa, Weaks-Dybvig, and Osdoby, 1983; and Davidovitch et al., 1980). From these studies, it has been found that the osteoblast plays a major role in bone remodeling and even has important roles in the function of osteoclasts.

**Osteogenesis**

Bone formation occurs by mesenchymal cells by one of two routes. First, as by direct development of bone from these cells, as found in the calvarium. This is also referred to as intramembranous ossification. Second,
a cartilage model precedes bone formation, where the proliferation of mesenchymal cells is followed by their differentiation into chondrocytes, which hypertrophy and calcify. The calcified cartilage is replaced by bone, which is then subject to remodeling. This type of bone formation is called endochondral ossification, and is frequently found in the long bones. Research has shown that even though these two modes of ossification are different, the two types of bone are not significantly different when compared together (Ham and Cormack, 1979).

Osteogenesis is a process that takes several months and involves both bone formation and resorption. The cell events involved in bone formation include the attraction of osteoblast precursor cells to the site of resorption by a process of chemotaxis. The stimulation of these precursors is followed by differentiation to mature cells which can synthesize bone proteins. Osteoblasts synthesize and secrete extracellular organic bone matrix, including Type-I collagen, osteocalcin, osteopontin, osteonectin, alkaline phosphatase, proteoglycans and the growth regulatory factors which are stored within the bone matrix (Meghji, 1992). Collagen is initially secreted from the cell surface in an organized fashion, so that the fibrils are orientated parallel to the long axis of the cells (Jones, Boyde and Pawley,
When the osteoblasts become embedded in the matrix, they become osteocytes by definition. Osteocytes may continue to produce a limited amount of collagen before becoming completely embedded. Most of the collagen, however, is produced by the active osteoblasts on the bone forming surface where they form a pavement-like sheet of closely packed cells (Meghji, 1992).

**Osteoblastic influence on osteoclasts**

Evidence indicates that the function of the osteoclast is regulated by cells of the osteoblast lineage (Vaes, 1988). The exact mechanism underlying osteoblastic regulation of osteoclasts is not well understood, although recent *in vitro* studies have suggested several inter-related mechanisms. First, osteoblasts may promote indirect resorption by degrading the non-mineralized surface osteoid layer of bone (Heath *et al.*, 1984b; Otsuka, Sodek and Limeback, 1984; Chambers, Darby and Fuller, 1985). This subsequently exposes the underlying mineralized matrix to the osteoclastic resorptive action. Second, data indicate that osteoblasts produce a short-range soluble mediator or mediators that directly activate
osteoclasts (Wong, 1984; McSheehy and Chambers, 1986b; Perry et al., 1987). Finally, regulatory osteoblast-derived growth factors may be sequestered in the bone matrix. The results of one study suggest that these factors may be liberated and activated by the proteolytic action of the osteoclast during osteoid removal (Oreffo et al., 1989). In addition, 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is known to be a potent stimulator of osteoclastic bone resorption. Mature osteoclasts, however, do not have receptors for 1,25-(OH)₂D₃, leading one to suspect that some type of interaction must be present between osteoblasts and cells with 1,25-(OH)₂D₃ receptors. Results from another study indicate that the effect of the hormone on osteoclast activity may be mediated through osteoblasts (McSheehy & Chambers, 1986b). Current evidence suggests that the osteoblast regulates both the formative and resorptive phases of the bone remodeling cycle. They are able to do so in response to both hormonal and mechanical stimuli. This study focused on the response of the osteoblast to mechanical stress.
Effects of mechanical stress on bone

Although the process of moving teeth through bone has been observed clinically, on a biological level, many questions as to how bone cells specifically respond to mechanical stimuli remain unanswered. A number of events occur in response to stress that is applied to the cell. First, when a mechanical stimulus is applied to the cell, there is a physical change in cell shape. This change can trigger cytoskeletal matrix interactions, which results in a series of cell responses. The close association with proteins present in the cell membrane (i.e. integrin), and inside the cell (i.e. talin, vinculin, and actin) lead to cytoskeletal matrix interactions because the cell shape has been altered. This leads to several different cascades of second messengers inside the cell.

Several investigators have identified the role of mechanical stress application and its influence on initiating various intracellular transduction cascades (Somjen et al., 1980; Binderman et al., 1988; Sandy et al., 1989a; Sandy et al., 1989b; Ngan et al., 1990; Sandy, Farndale, and Meikle 1993). These include the activation of phospholipase A₂ (Binderman et al., 1988), the interaction of interleukin-1β and its effect on Prostaglandin E (PGE) and
adenosine 3', 5'-cyclic monophosphate (cAMP) production (Ngan et al., 1990; Somjen et al., 1980), and involvement with phosphatidylinositol pathways (Sandy et al., 1989b). Mechanically deformed tissues have been shown to have many cellular effects associated with orthodontic tooth movement, which may be caused by cytokine production (Sandy, Farndale, and Meikle 1993). It has also been hypothesized that many types of cell messengers could mediate the effects of mechanical deformation. Two main examples include cAMP and the phosphatidylinositol pathway. In both cases, as the cell is stretched, stimulatory agonists bind with receptors in the cell membrane which interact with respective G-proteins. This G-protein complex causes the activation of adenylate cyclase, which forms cAMP from ATP. Furthermore, cAMP activates protein kinase A, which phosphorylates proteins, and ultimately leads to a cell response (Sutherland and Rall, 1958).

The second cell messenger, the phosphatidylinositol pathway, can lead to a cascade of events which eventually causes a sustained cell response and an increase in the levels of intracellular calcium. In this pathway, phosphodiesterases (a phospholipase C) cleave phosphatidylinositol bisphosphate into diacylglycerol (DG) and inositol triphosphate (IP3). DG remains in the cell membrane and activates protein
kinase C, which phosphorylates proteins and leads to a sustained cell response. IP₃ mobilizes Ca²⁺ from endoplasmic reticulum stores and initiates the early response in the cell. This also leads to a sustained response (Streb et al., 1984; Streb et al., 1983; Hokin and Hokin, 1953) within the cell.

These cascades have been identified, however their precise relation to application of mechanical stress has not completely been determined. Studies using adult rat and avian bone have been used as models for application of mechanical stress, and have displayed osteoblastic proliferation, inducing new bone formation (Pead, Skerry, and Lanyon 1988; Miyawaki and Forbes, 1987). In a similar manner, the application of mechanical stress to bone cells grown in tissue culture leads to increases in cell proliferation (Burger and Veldhuijzen, 1993) and elevated levels of cAMP (Rodan et al., 1975a; Sandy et al., 1989; Burger and Veldhuijzen, 1993). Mechanical stress has also been found to affect alkaline phosphatase (AP) levels within bone (Rodan and Rodan, 1984). Low strain levels have been found to increase AP production (Burger and Veldhuijzen, 1993), while somewhat higher levels have been found to decrease AP production in these cells (Inoue et al., 1993; Burger and Veldhuijzen, 1993). Mechanical stress on bone cells has also been found to have increased incorporation of 3H-
thymidine in the cells, a marker for increased cell division (Shimshoni et al., 1984), enhanced synthesis of prostaglandin E$_2$ (PGE$_2$) (Somjen et al., 1980; Yeh and Rodan, 1984; Burger and Veldhuijzen, 1993), and elevation of inositol phosphates (Sandy et al., 1989). Part of this study will look at PGE$_2$ production in response to mechanical deformation of the osteoblastic cells.

**Prostaglandin E$_2$ (PGE$_2$)**

PGE$_2$ is an important cell metabolite in the process of bone resorption. It is synthesized from arachidonic acid (ARA), which commonly exists in an esterified form within mammalian cell membrane phospholipids. The arachidonic acid metabolic pathway has two major branches: the cyclooxygenase and the lipoxygenase pathways. The lipoxygenase pathway results in the production of hydroxyeicosatetraenoic acids and leukotrienes. In contrast, the cyclooxygenase pathway ultimately leads to the synthesis of prostaglandins, thromboxanes, and prostaclins. Cyclooxygenase exists within cells in an active state and does not require enzymatic cleavage or phosphorylation for activation. The existing amount of cyclooxygenase, or that which has been newly synthesized within the cell acts as a regulator,
limiting the reaction. This enzyme undergoes irreversible inactivation, or spontaneous oxidation. Research indicates that PGE is synthesized after almost 1300 cycles of ARA metabolism, primarily due to this spontaneous oxidation phenomenon seen with cyclooxygenase (Smith and Lands, 1972; Hemler and Lands, 1980). Studies suggest that the half-life of one form of cyclooxygenase is very short; less than 10 minutes (Wu et al., 1988; Fagan and Goldberg, 1986). This means that in order for a cell to sustain a prolonged PGE secretory response, de novo synthesis and replenishment of cyclooxygenase are required. As PGE is synthesized, it is simultaneously released and is not stored intracellularly prior to secretion. Most cells release a small amount of PGE as a byproduct of membrane transduction events and cellular activation. It has been found that blocking this membrane transduction signal by inhibiting PGE₂ synthesis can alter or block certain responses that are triggered by various agonists (Offenbacher, Heasman and Collins, 1993).

The effects of PGE on target cells are receptor mediated. The PGE receptor is a small membrane associated protein (Sibley et al., 1987) which utilizes G proteins as intermediates in transmembrane signaling pathways including the adenylate cyclase system (Negishi, Ito and Yokohama, 1988;
Sonnenburg, Zhu and Smith 1990; Negishi, Ito and Hayaishi, 1989). It therefore follows that when the PGE receptors are occupied, this triggers G protein coupling, and adenylcyclase activation, with subsequent increased intracellular levels of cAMP (Hakeda et al., 1987; Farndale et al., 1988; Civitelli et al., 1988; Kawaguchi et al., 1995).

**PGE$_2$ and its effect on bone cells**

Prostaglandin E$_2$ is an important cell metabolite in the process of bone resorption and has been found to both inhibit and stimulate bone resorption (Raisz and Martin, 1983). PGE$_2$ has been widely studied and has been shown to stimulate new bone formation *in vivo* and to increase collagen synthesis in bone organ cultures at low concentrations (Norrdin et al., 1990) or in the presence of cortisol (Raisz and Fall, 1990). In contrast, high concentrations of PGE$_2$ have been found to inhibit collagen synthesis in both clonal osteoblastic cell line Py1a and organ cultures, especially in the presence of insulin-like growth factor (Raisz and Fall, 1990; Raisz et al., 1993). Research indicates that PGE$_2$ can also stimulate bone resorption when in organ cultures (Klein and Raisz, 1970; Dietrich, Goodson, and
Raisz, 1975; Rifkin, Baker, and Coleman, 1980). This effect has been found to be associated with increased levels of cAMP as well as elevated numbers of osteoclasts, which show increases in activity and mobility (Chambers and Dunn, 1983). The findings from these studies indicate that PGE could be used as an indicator when applying mechanical stress to bone cells because it can be present during both bone formation and bone resorption.

**Studies involving mechanical perturbation of cells**

Many *in vitro* studies have been conducted that have used various mechanical devices to apply mechanical stress to cells, and determine their responsiveness in an attempt to answer the question, “How do cells transduce mechanical stress?” An early experiment was designed to deliver compressive forces to long bones using a piston device (Rodan et al., 1975a; Bourret and Rodan, 1976). In another study, rabbit aorta cells were seeded on an elastic membrane and stretched with a mechanical cycling machine. It was found that this stretch application led to large increases of cellular metabolites including: collagen, hyaluronan, and chondroitin 6 sulfate. A different team failed to detect an effect on DNA synthesis or other cell
metabolites (Leung, Glagov, and Mathews, 1976) in response to stretch. In chondrocytes from chick embryo, another team found increased levels of glycosaminoglycan and a decrease in protein and collagen synthesis (Lee et al., 1982). Others used a “mechanical perturbation machine” in which culture dishes with flexible bottoms were stretched over 12 watchglass domes (Norton et al., 1995; Norton et al., 1992, Andersen and Norton, 1991; Andersen, Pedersen, and Melsen, 1991). A piston was attached to metal arms that were connected to an upper table, which was capable of raising and lowering this table. The culture dishes were attached to the upper table with wax and were raised and lowered over watchglass domes below them. This applied an intermittent stretch to the cells on the bottom of the culture dishes. Marked differences were observed in cell shape when stretching fibroblasts. These studies have revealed the findings from several investigations reported in the literature, where different types of cells were stretched. It is possible that these data could be applied to additional studies involving stretching of osteoblast cells.
Stretching of osteoblast cells

With respect to bone cells, several investigations have examined cell changes after using an orthodontic jackscrew appliance attached to the sides of the plastic culture dishes (Binderman and Cox, 1977; Somjen et al., 1980; Harrell, Dekel, and Binderman, 1977). Although the force applied was unknown, findings indicated that there were increases in the levels of PGE₂. In another study, osteoblastic ROS 17/2.8 cells were seeded on a collagen sheet, which then underwent known tensile or stretching forces over a short period of time (Yeh and Rodan, 1984). These cells also showed increases in the quantity of PGE₂ production (Rodan, Yeh, and Thompson, 1989; Mostafa, Weaks-Dybvig and Osdoby, 1983).

Others used an expandable "table-like" apparatus designed to pull at cranial sutures (Meikle et al., 1979; Meikle, Sellers and Reynolds, 1980; Meikle et al., 1982; Meikle, Heath and Reynolds, 1984). They observed many cell events including the identification of the phenotypical characteristics of collagen and the pathways to metalloproteinase synthesis. Others studied DNA production in rat osteoblasts under these similar conditions (Hickory and Nanda, 1987). Changes in cell shape were
observed after application of tensile forces in mouse interparietal sutures by using an orthodontic wire pulling device (Yen et al., 1990). Another team observed cell changes by using a variation of this expandable table by stretching culture dishes with distortable polytetrafluoroethylene (PTFE) membranes (Hasegawa et al., 1985). Known forces were applied to the dishes that were then deformed over dome shaped glass to a specified calculated strain. Frequency of application of these forces could also be varied.

One study cultured osteoblasts from the calvarium onto PTFE membranes and subsequently subjected these cells to mechanical deformation using this device (Sandy et al., 1989a; Sandy et al., 1989b). They found that osteoblasts had an increase in cAMP and inositol phosphates in response to stress application. Thus they hypothesized that a probable phosphoinositol pathway exists that responds to applied stress in these cells. Others looked at embryonic rat calvaria cells and applied an unquantified level of stress to these cells (Binderman et al., 1988). They observed that stress application led to an increase in arachidonic acid which increases prostaglandin production. Another study looked at MC3T3-E1 osteoblastic and PDL cells, applying both positive and negative pressures
intermittently (Saito et al., 1991). Findings indicated that PGE increased for both positive and negative pressures, but a substantial increase was found when applying positive pressure. Interleukin 1 beta (IL-1β) was added to the cultures and negative pressure was applied. This caused PGE levels to decrease significantly. PGE levels also increased in PDL cells when positive pressure was applied with addition of IL-1β. In the same study, tension was applied to PDL cell cultures on PTFE membranes. They found that levels of PGE increased with stress application and discovered that IL-1β further magnified this increase.

Ion channels and their relation to cellular events

The membranes of all cells contain ion channels, that permit the passage of specific ions in and out of the cell. These channels are important as they help regulate many biologic functions. Ion channels are divided into several groups, based upon the type of stimulus needed to activate the channel. The major groups are: voltage gated, ligand gated and mechanosensitive (stretch) ion channels. Voltage gated channels have been found to open or close when subjected to changes in transmembrane
potential. In this type of ion channel, specific domains are present in the channel proteins which cause them to undergo a conformational change in response to voltage. Ion channels are also selective for the types of ions which they permit to enter or leave the cell. Ligand gated ion channels also have similar domains imbedded in the channel protein, where specific ligands can bind and elicit a change in the structure of the channel protein. Again, this influences the channel activity and regulates the flow of particular types of ions in and out of the cell. Finally, there are mechanosensitive ion channels which respond to structural changes in the cell membrane. Once again, special domains within ion channel proteins modulate the changes in ion channel activity.

**Mechanical deformation of ion channels**

Various investigators have examined different types of cells and their response to mechanical deformation (see reviews by Sachs, 1988; Morris, 1990). These include cochlear hair cells (Ohmori, 1984), renal proximal tubule cells (Sackin, 1989), smooth muscle (Kirber, Walsh, and Singer, 1988), and skeletal muscle cells (Guharay and Sachs, 1984). The following
is a list of selectivity and conductances for stretch activated channels that have been found in various cell tissues (Sachs, 1988). To the left, the tissue type is listed, with the column to the immediate right of this column identifying the ionic selectivity, either cation or anion selective. The next column identifies the conductances for the various ion types in pico Siemens (pS), with the right most column listing the references.

<table>
<thead>
<tr>
<th>Tissue studied</th>
<th>Ionic selectivity</th>
<th>Cond. in (pS)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick muscle</td>
<td>Cation, K&gt;Na&gt;Li</td>
<td>35/Na</td>
<td>70/K Guharay &amp; Sachs, 1984</td>
</tr>
<tr>
<td>Xenopus muscle</td>
<td>Cation</td>
<td>50/Na</td>
<td>----- Brehm et al., 1984</td>
</tr>
<tr>
<td>Snail heart</td>
<td>Cation, K&gt;Na</td>
<td>33/Na</td>
<td>85/K Brezden et al., 1986</td>
</tr>
<tr>
<td>Frog lens</td>
<td>Cation, K&gt;Cs&gt;Na&gt;Li</td>
<td>28/Na</td>
<td>30/K Cooper et al., 1986</td>
</tr>
<tr>
<td>Rat endothelium</td>
<td>Cation, Ca&gt;K&gt;Na&gt;Cs</td>
<td>40/Na</td>
<td>56/K Lansman et al., 1987</td>
</tr>
<tr>
<td>Frog oocytes</td>
<td>Cation, K=Cs&gt;Na&gt;Li</td>
<td>52/Na</td>
<td>73/K Yang &amp; Sachs, 1986</td>
</tr>
<tr>
<td>Chick hair cells</td>
<td>Cation, Li&gt;Na&gt;K&gt;Cs</td>
<td>-----</td>
<td>50/Cs Ohmori 1984</td>
</tr>
<tr>
<td>Frog smooth muscle</td>
<td>Cation</td>
<td>55/Na</td>
<td>----- Kirber et al., 1987</td>
</tr>
<tr>
<td>Frog kidney tubule</td>
<td>Cation, K&gt;Na</td>
<td>26/Na</td>
<td>47/K Sackin 1987</td>
</tr>
<tr>
<td>Turtle colon epitheli</td>
<td>Cation, K&gt;Na</td>
<td>17/K</td>
<td>----- Richards &amp; Dawson 1986</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>Cation</td>
<td>20/Na</td>
<td>----- Falke &amp; Misler unpublished</td>
</tr>
<tr>
<td>Snail neurons</td>
<td>Cation, K&gt;Na</td>
<td>32/Na</td>
<td>----- Sigurdson et al., 1987</td>
</tr>
<tr>
<td>Tobacco protoplasts</td>
<td>Anion</td>
<td>-----</td>
<td>200/Cl Falke et al., 1987</td>
</tr>
<tr>
<td>Yeast protoplasts</td>
<td>Cation</td>
<td>40/K &amp; 70/K</td>
<td>----- Martinac et al., 1987 &amp; Gustin et al., 1987</td>
</tr>
<tr>
<td><em>E. coli</em> protoplasts</td>
<td>Anion</td>
<td>-----</td>
<td>970/Cl Martinac et al., 1987</td>
</tr>
</tbody>
</table>

The earliest study of stretch activated ion channels (Guharay & Sachs, 1984) was conducted using chick skeletal muscle in tissue culture. At the same time, others reported the same kind of stretch activated ion channel
in embryonic *Xenopus* muscle (Brehm *et al.*, 1984). The fundamental observation of the first study was that the open-channel conductance was essentially independent of membrane tension. This study helped to rule out the model of transduction where simply the channel is normally closed, but under stretch, the channel is pulled open. Additionally, they obtained excised patches of membrane and recorded ion channel activity over long periods of time. They also determined that although an ideal channel would be either open or closed, that in reality, there are multiple conducting states present. This idea of multiple conducting states for ion channels was reviewed in another paper looking at a variety of channels (Auerbach and Sachs, 1984).

For animal cells, stretch activated channels all show cation selectivity. Based on reversal potential measurements, there appears to be two kinds of channels. The first type has been shown to have a reversal potential highly selective for potassium. This type of channel has been reported in snail heart (Brezden *et al.*, 1986) and in frog kidney tubules (Sackin, 1987). The second type occurs in frog (Cooper *et al.*, 1986) and chick cells (Guharay & Sachs, 1984), where the reversal potentials are close to zero when in physiologic saline.
In frog lens epithelia (Cooper et al., 1986), the current-voltage curves of the stretch activated channels found in these cells show a strong inward rectification with reversal potentials near zero. Also the selectivity of this channel was found to be modulated by extracellular calcium levels, where in low calcium (50 \( \mu M \)), the channel was more permeable to K\(^+\) than to Na\(^+\). When this quantity was raised to 2 mM, the conductance to both K\(^+\) and Na\(^+\) was greatly decreased and the I/V curves for both ions became indistinguishable.

Further review of the literature of stretch activated ion channels has helped to determine some additional properties inherent in these channels. Two studies looking at yeast protoplasts (Martinac et al., 1987; Gustin et al., 1987), observed that stretch activated channels can display multiple conductance states. When observing the “open” channel in a different study using frog oocytes (Yang & Sachs, 1986), a subconductance state was identified. This is where the channel is open and the current is “noisy”. Here, the “noise” was observed to consist of both slow drifts and small step-like displacements. In these records, however, a clear population of subconductance amplitudes has not been identified.

Another study observing E. coli protoplasts (Martinac et al., 1987) was
found to be anion selective for chlorine. The investigators also found that the probability for the channel to be open ($P_{\text{open}}$) was dependent on membrane voltage and indirectly dependent on the current. However, they found that it was possible to reverse the direction of the current without producing major changes in the kinetics under certain ionic conditions. This implied that the current itself is not a source of energy. In addition, although the most characteristic property of stretch activated channels is the dependence on tension or stretch, the channels were also found to be voltage dependent, where depolarization has been shown to increase the $P_{\text{open}}$.

Finally, in rat endothelium (Lansman et al., 1987), channel activation was found to lag the stimulus or suction applied to the patch by 5 ms. As the suction was removed, the response appeared to decrease within 10 ms.

**Studies involving ion channels in osteoblastic cells**

Many studies have looked at the effect of mechanical perturbation on ion channel activity in mineralized tissue, such as osteoblastic cells. The transduction of mechanical stress into a biochemical signal can occur via stretch sensitive ion channels, which have been described in the osteoblast
cell membrane (Duncan and Misler, 1989). Other studies show that several different types of responses occur when bone cells are perturbed. An example is that levels of some second messengers (i.e., cAMP and PGE$_2$) have been shown to increase in response to stretch, which in turn have produced alterations in membrane potential (Ferrier et al., 1988; Chow et al., 1984).

Transmembrane potential is the electrochemical difference that exists between the two sides of the cell membrane. Ion channels are important mechanisms that help to regulate and change the membrane potential. These channels are tunnel-shaped transmembrane proteins that serve as selective conductive pathways for ions across the plasma membrane and across membranes surrounding intracellular organelles (Hille, 1984). The opening and closing of these conduction pathways may be controlled by differences in the concentration of extra- and intracellular substances, voltage, and by mechanical stress of the membrane (Morris, 1990). Finally, several substances have been found to stimulate stretch activated ion channels. These include: neurotransmitters, hormones, autocrine substances, and second messengers (Chesnoy-Marchais and Fritsch, 1989).

Two events occur within the cell when more than one of the same type
of ion channel opens. First, there may be a change in membrane potential, which could influence the flow of other ions through different channels. As a result of this change, other ion channels may open or close which could alter the driving force for the ions that would pass through these already open channels. Others have found that osteoblastic membrane characteristics permit passive transmembrane transport of a given type of ion, which is coupled to the transport of other ion types (Ypey et al., 1992). This has also been found to modulate changes in membrane potential.

Secondly, changes in the number of open ion channels causes a change in the intracellular concentration of electrolytes. This may, in turn, act as a signal to the cell to change its functional activity. Several researchers have studied various changes in cell function when Ca\textsuperscript{2+} ions enter the cell. This influx of Ca\textsuperscript{2+} ions may possibly control protein secretion (Guggino et al., 1989), fluid secretion (Petersen and Maruyama, 1984), cell movement (Zaidi, Mac Intyre and Datta, 1990), or other metabolic processes.

Many investigators that have studied the importance of ion channels in bone cell functions (Chesnoy-Marchais and Fritsch, 1989; Chesnoy-Marchais and Fritsch, 1988; Davidson, Tatakis, and Auerbach, 1990; Ferrier et al., 1987; Grandolfo et al., 1990; Sims and Dixon, 1989; Sims, Kelly, and Dixon,
1991; Ypey et al., 1988) by using the patch-clamping technique (Hamill et al., 1981; Neher and Sakmann, 1976). This is a powerful technique that permits the measurement of microscopic picoampere ($10^{-12}$ A) currents through single ion channels in isolated patches of cell membrane. The majority of the ion channels in osteoblast cells have been found to be voltage activated (Ravesloot, et al., 1989), but some are sensitive to stretch in the cell membrane (Duncan and Misler, 1989; Duncan, Hruska and Misler, 1992; Duncan and Hruska, 1994; Davidson, Tatakis, and Auerbach, 1990; Davidson, 1993). This stretch sensitivity in bone cells may imply a mechanism for bone adaptation to changes in mechanical loading.

Other investigators have monitored changes in osteoblastic membrane potential in response to ion channel activation. In rat osteoblasts, as well as in rat ROS 17/2.8 and UMR-106 cells, others have discovered an array of different types of voltage-gated Ca$^{2+}$ channels (Guggino et al., 1988; Yamaguchi et al., 1987; Chesnoy-Marchais and Fritsch, 1988; Grygorczyk, Grygorczyk and Ferrier, 1989; Karpinski et al., 1989; Duncan and Misler, 1989). One study has identified a depolarization activated, tetrodotoxin-sensitive Na$^+$ channel (Chesnoy-Marchais and Fritsch, 1988), and another identified a voltage-dependent, high-conductance anion channel in Py1a rat
osteoblast-like cell lines (Davidson, 1991). In rat osteoblasts, ion channels were found to have both transient and sustained voltage-controlled conductances (Ypey et al., 1988; Chesnoy-Marchais and Fritsch, 1988). Of the identified K⁺ channels present in osteoblasts, one study has identified a Ca²⁺-activated voltage dependent K⁺ channel (Ypey et al., 1988; Ypey et al., 1992). In the human osteoblast-like osteosarcoma cell line G292, others have detected diverse classes of K⁺ -selective channels (Davidson, Tatakis, and Auerbach, 1990; Davidson, 1993). These studies show that there may be numerous types of ion channels present in different osteoblast cells.

**Mechanosensitive ion channels in bone cells**

Mechanical stimulation of osteoblastic cells in vitro has been shown by many investigators to result in increased metabolic activity, due to a change in membrane potential (Chow et al., 1984; Heath et al., 1984a & 1984b; Edelman, Fritsch and Balsan, 1986; Ferrier and Ward, 1986; Fritsch, Edelman and Balsan, 1988; Ngan et al., 1988). One group using UMR-106 osteogenic sarcoma cells used the cell-attached patch-clamp recording and found evidence of two ion channels (Duncan and Meisler, 1989). The first
channel was capable of carrying inward Ba\textsuperscript{2+} currents at membrane potentials near to or moderately depolarized from the resting membrane potential of the cell. In addition, these channels would also be expected to carry Ca\textsuperscript{2+} currents. The second channel was found to be voltage-insensitive, but stretch-activated. At a given current voltage, this channel carried inward currents of similar magnitude when physiologic saline or near isotonic KCl was present at the extracellular surface. This suggested that the channel was poorly selective among cations. Another team found similar activity and an increased activation to PTH in mechanosensitive ion channels of the same UMR-106 osteosarcoma cell line (Yamaguchi et al., 1989). In addition, others applied PTH to UMR-106 osteosarcoma cells in concentrations which had been found to effect other cell functions (Duncan, Hruska and Misler, 1992). They found a resultant increase in activity of a stretch activated non-selective cation channel by observing cell-attached patches of the cell membrane. This effect was accompanied by a small increase in single channel conductance. Their observations suggest that the non-selective cation channel might be a locus at which the actions of membrane deformation and PTH converge. Some members of this same team later conducted an additional experiment using the same cell line
(Duncan and Hruska, 1994). They demonstrated that application of chronic
intermittent mechanical stretch modulated the stretch activated cation
channel in these cells. This was done by increasing the sensitivity of the
channel to stretch and the average number of open channels. Chronic
stretch was also shown to increase the whole cell conductance, which was
due to the changes in stretch activated cation channel activity. This was
hypothesized as they found that blocking the channel reversed the whole cell
conductance change.

In the G292 human osteoblast-like osteosarcoma cell line Davidson
and colleagues detected several stretch activated ion channels (Davidson,
Tatakis, and Auerbach, 1990; Davidson, 1993). These channels were found
to have at least three types of mechanosensitive ion channels. They applied
negative pressure after obtaining cell attached patches, and recorded
conductances of 20, 60 and 160 pS. They found that the large conductance
channel (160 pS) was K⁺ -selective and showed a similar kinetic behavior to
the Ca²⁺ -activated, voltage gated potassium channel that was previously
identified in other osteoblast cells (Dixon, Aubin and Dainty, 1984; Ypey et
al., 1988; Ravesloot et al., 1990). The 60 pS channel was found to be non
selective for cations, and the 20 pS channel was found to have conductance
properties similar to that of the Ca^{2+}-conducting cation channel found in UMR-106 osteoblastic cells (Duncan and Misler, 1990). Finding these channels and identifying their associated properties led them to hypothesize that a hyperpolarizing current, mediated in part by these three channels, may be associated with the early cell events during mechanical loading of bone cells. Further investigations are still required to determine the precise relationship between channel activation and bone remodeling.

Much of the information obtained from these early studies was important because it has helped to lay the foundation for identification of the properties found in stretch-activated channels present in bone cells, and their potential importance to the process of bone remodeling. As discussed, there are many cell events that take place within the osteoblasts when tensions and stresses are applied to the teeth and surrounding bone during orthodontic tooth movement. Prostaglandin production can be used as one marker or identifier and has been shown to increase when these cells undergo stretch.

At the level of the cell membrane, osteoblasts have been found to have many types of ion channels, several of which have been found to be responsive to stretch activation. These findings were taken into
consideration in planning and conducting the experiments in this investigation. This study focused primarily on ion channel activity in G292 osteoblastic-osteosarcoma cells to help give insight to some of the characteristics of the mechanosensitive ion channels in bone cells when forces are applied to teeth during orthodontic tooth movement.
HYPOTHESIS

The hypothesis of the proposed research is that mechanical perturbation of the G292 osteoblast-like cells will result in:

1) increased levels of specific prostaglandins (especially $\text{PGE}_2$)

and

2) increased activity of the mechano-sensitive ion channels present in these cells.

It is also hypothesized that this response will vary with the frequency and/or duration of the mechanical stimulus.
SPECIFIC AIMS

Specific Aim 1: Determine the optimal frequency and duration of mechanical perturbation (stretch), that would produce a metabolic effect in G292 osteoblast-like osteosarcoma cells (a well characterized human cell line): i.e. alterations in prostaglandin production and/or cell number.

Specific Aim 2: Identify the mechanosensitive ion channels in G292 cells and characterize the biophysical properties in cells that had been stretched.
General Experimental Design

The relevance of the proposed research was to obtain a better understanding of the cell mechanisms present in bone which respond to light, mechanical forces. The aim of this study was to simulate the light forces desired in a clinical setting to produce ideal orthodontic tooth movement. The first step was to culture a well-characterized line of human osteoblast-like osteosarcoma cells (G292) on stretchable polytetrafluoroethylene (PTFE) membranes in separate dishes. These cells were selected as an in vitro model of alveolar bone cells or osteoblasts.

The experimental process consisted of growing cells to confluence and seeding them on flexible Petriperm® dishes (Norton et al., 1992). Cell adherence was tested initially to determine if addition of a substrate to these dishes was required for cell attachment. The cells were separated into control and experimental cells, and then the experimental cells were subjected to a stretching force with differing frequency and duration. Next, the supernatant from each dish of cells was extracted and prepared for gas chromatographic mass spectrometry (GC-MS) analysis of specific prostaglandin levels. In addition, cell counts were obtained by using a
Coulter Counter® to determine whether cells were lost as a result of being stretched.

The cells were divided into six experimental groups and were subjected to mechanical perturbation. After this, patch clamping was performed on the cells. Cell electrophysiology was monitored by recording ion channel activity and observing the changes present in membrane currents using these recording techniques. The patch-clamp technique was used because it has been identified as an established method to accurately study bone cell physiology. Also, it is an important method to investigate ion channel conductances across single channels of intact living cells. The results obtained from this investigation help give insight as to what happens at the cell level when osteoblasts are subjected to stretch in vitro. These cell responses have provided an understanding of in vitro mechanisms that may be relevant to physiological bone remodeling that occurs during orthodontic tooth movement in vivo.
MATERIALS AND METHODS

Human osteoblast osteosarcoma G292 cells (no. CRL 1423) were obtained from American Type Culture Collection (ATCC) (Rockville, MD.). This cell line was taken from a primary bone tumor (osteosarcoma) of a 9 year old caucasian female. The cells were frozen at passage 13 when received. I chose to experiment with these cells because they have an osteoblastic phenotype, and their electrophysiological properties have been described extensively in the literature (Davidson et al., 1989; Davidson, Tatakis, and Auerbach, 1990a; Davidson et al., 1990b; Davidson, 1993).

Cell culture

The following protocol was used in all experiments involving cell culturing and seeding. Cells were thawed and seeded to prepare stock cell cultures. All preparations were made under aseptic conditions. Subcultures of early passage cells (passages 15-21) were seeded into stock flasks and grown to confluence in McCoy's 5A medium supplemented with 10% fetal
bovine serum (FBS) and antibiotics (100 units of penicillin g/ml, and 100 μg of streptomycin/ml). These cells were maintained at 37°C in a humidified Cell Star incubator with a controlled mixture of 5% CO₂ added. Media changes took place every other day (at approximately 48 hour intervals) and the G292 cells were allowed to proliferate to a confluent monolayer. These cells were divided and separated with 0.25% trypsin in physiologic saline and detached from the stock flasks over 5 minutes. Subsequently the cells were suspended by invert pipetting with 8 ml of McCoy’s 5A media, 10% FBS, and antibiotics as previously described, and centrifuged at 1200 rpm for 5 minutes using a Damon/IEC Division IEC PR-J centrifuge at 25°C. The cells were then thoroughly mixed and seeded onto Petriperm®, flexible bottom dishes from the original stock flasks to obtain a semi-confluent state. Cell densities were determined by placing cells in trypan blue, mixing, and placing 10 μl on each side of a hemacytometer slide, then counting numbers of cells present within a specified grid. Several recordings were taken for each seeding and averages taken to determine appropriate cell concentration. The final desired cell concentration was 5 x 10⁴ cells/ml.
Mechanical stimulation of cells

Mechanical strain was applied to the cells by flexing the flat membrane at the bottom of the dish to a curved configuration by contacting a silicone-lubricated convex curved watchglass (Hasegawa et al., 1985; Norton et al., 1992). Strain levels (ε) approximating those levels corresponding with the force required to perform bodily orthodontic tooth movement have been previously calculated for this experimental model (Andersen, Pedersen and Melsen, 1991; Andersen & Norton, 1991; Norton et al., 1992). This level of percent strain was found to be 1% ε. The watchglasses were custom ground for radius and height above a horizontal plane to match these strain levels, using the equations in Figure 2. Twelve domes were mounted on the main lower plexiglass platform while the upper platform was weighted and can be lifted from the lower. The watch glasses were lubricated with silicone to reduce friction and to insure a uniform stretch application to the dishes. When the upper platform lifted from the watch glass domes, tensile stresses on the flexible membrane of the culture dishes were relieved. The mechanical perturbation of all cells was performed inside an incubator with
conditions similar to that used during the culturing and seeding of these cells. All dishes from the same individual experiment underwent metabolic assays to detect levels of PGE$_2$ and cell number. Comparisons were made between stretched cells and controls.

**Adherence tests**

I first wanted to identify if stretching cells led to a significant loss of cells on the bottom of the flexible dishes. If a significant loss of cells was found, then it would be important to include an additional substrate to enhance the adherence of the cells to the flexible dish bottom. This variable could introduce a source of error into the study. Cells were cultured and seeded to near confluence on flexible Petriperm$^\text{R}$ dishes, and placed in an incubator for two days to permit the cells to adhere. Cells were stretched using a timer-controlled device (Andersen & Norton, 1991) (Figure 1), which is capable of stretching the membranes of 12 PTFE disks concurrently. Cells were trypsinized and added to 8 ml of McCoy’s 5A media, then centrifuged and 1 ml of PBS (Physiological biologic saline) was added. One hundred $\mu$l
of this solution was added to 20 ml of Hematal in vials, shaken thoroughly, and placed in a Coulter Counter® (Coulter Electronics, Hialeah, FL). Counts were taken twice at an amplification of 4 and an aperture current of 4 mA. Control cell numbers were compared to numbers of cells that had been perturbed to determine if cells were being lost. Coulter counting was also used later in the metabolic studies to determine the amount of prostaglandin produced when compared to cell number. Cell retention was important because it helped to verify that the cells that remained fixed to the bottom of the dish were in fact, being stretched and not dislodged.

For the adherence tests, 13 experiments were conducted comparing stretched cell numbers to control cell numbers (Table I). The arithmetic means of each of the stretch and control cells were calculated for each of the 13 experiments. A paired t-test was conducted to detect whether there were significant losses in cells between stretch and control groups (Table II).

**Metabolic Assays**

Prostaglandins were extracted and separated in a manner similar to that previously described (Payne, Peluso, and Nichols, 1993). The
osteoblastic culture supernatants were supplemented with [3,3,4,4,-2H]-PGE$_2$ (98% atoms as $^2$H form, 100 ng, Cayman Chemical Co., Ann Arbor, MI, USA) as an identifying marker and adjusted to pH of 3.0 with formic acid (70%). Culture supernatants were applied to octadecyl (C$_{18}$) bonded-silicic acid columns (Supelclean SPE Tubes, 3 ml, Supelco, Inc., Bellefonte, PA, USA) mounted on a vacuum manifold, as described by Luderer, Riley, and Demers, 1983). Columns were washed with PBS (pH 3.0, 2 ml) and followed by adding 25% methanol-water (2 ml). The metabolites were then eluted with 100% methanol and dried overnight under a vacuum. The dried residues were reconstituted in 1% formic acid in water (2 ml) and extracted with chloroform (2 ml x 3). The collected organic extracts were then dried under nitrogen and stored at -20°C.

The supernatant samples were then treated as previously described (Waddell, Blair, and Wellby, 1983; Payne, Peluso, and Nichols, 1993), in preparation for analysis by capillary gas chromatography-mass spectrometry (GC-MS). All samples were first treated overnight with methoxylamine hydrochloride (50 μl). The samples were then dried with nitrogen, and dissolved in acetonitrile (30μl). This was followed by subsequent treatment with pentafluorobenzyl bromide (35% v/v in acetonitrile, 10μl) and
diisopropylethylamine (10μl). This solution was then heated at 40°C for 15 minutes and evaporated under nitrogen. The residue was then treated with bis-trimethylsilyltrifluoroacetamide (50μl) and allowed to stand for 4 days. GC-MS analysis was performed using the Hewlett Packard 5890-gas chromatograph interfaced with a 5988A-mass spectrometer. The materials and methods for this procedure were similar to those used previously (Payne, Peluso, and Nichols, 1993). In addition, cell numbers were counted as previously mentioned to associate prostaglandin production with actual cell counts.

Cell preparation for electrophysiological studies

In these experiments, the osteoblastic G292 cells were seeded from stock flasks to Petriperm® flexible dishes to achieve similar semi-confluent monolayer states. All dishes containing cells were incubated under identical conditions for 2 days prior to beginning the experiment. I had previously determined that waiting 2 days after seeding was an optimal time to allow the cells to properly attach to the flexible membrane of the dish. Immediately before dividing the dishes into stretch and control groups, all dishes were
reexamined microscopically to check for similarity in degree of confluence of the monolayer. Dishes were marked for identification and the upper components of the dishes were sealed to the lower components with wax. All dishes were placed in the cell perturbation machine under the same incubation conditions. I conducted six complete experiments where each experiment contained a total of 12 dishes (6 experimental dishes which would undergo stretch and 6 controls). A 1 kg weight was placed on the table above the dishes to insure continuous stretch application. Dishes were categorized into groups according to time of stretch application: 1 hour, 24 hours, and 48 hours.

Immediately after mechanical perturbation, the supernatant was removed from each dish and four 1 cm x 0.5 cm strips were excised from the flexible culture dish bottom using a new #15 scalpel blade. These strips were placed in separate dishes containing McCoy's 5A media for temporary storage, and then transferred to the perfusion chamber. This chamber rested above the microscope and bathed the cells at room temperature in a recording media consisting of calcium free saline (ECS) [in mM: 145 NaCl, 5 KCl, 1 MgCl₂, and 10 HEPES/ NaOH (pH 7.4)]. CaCl₂ (1.5 μl of 1M) was added per ml of ECS solution to aid in the ability to obtain patches.
**Electrophysiological Studies**

Osteoblastic cells were studied using the patch-clamp electrophysiologic technique (Hamill et al., 1981). In this technique, glass micropipettes were prepared from 1-2 mm diameter borosilicate glass capillary tubes (Drummond Scientific, Broomall, PA). The tubes were electrically heated, drawn, and separated to form two pipettes using a Flaming/ Brown Micropipette pulling machine (Sutter Instrument Co., Model P-87). After heating and separation, all pipettes had steep tapers at their tips, with opening diameters ranging from 0.5 to 1\(\mu\)m. The pipette shanks were subsequently coated near the tip with Sylgard (Dow-Corning, Midland, MI) and heat cured with a heating gun. The Sylgard coating was placed in close proximity to the tip of the pipette to reduce the pipette-bath capacitance and to form a hydrophobic surface. This served to reduce the background noise which is created as current flows through the pipette. This background noise exists because the bath solution and the uncoated, hydrophillic tip of the pipette form a large capacitor, due to an increased separation of charge. The pipette tips were then fire polished with a microforge (Narishige
The pipettes were back filled with a solution containing an intracellular-like saline potassium-rich solution (ICS) [in mM 5 NaCl, 145 KCl, 1 MgCl₂, 0.02-4 CaCl₂, 10 EGTA and 10 HEPES/ KOH (pH 7.4)]. 1.75 μl of 1M CaCl₂ was also added per ml of ICS solution to aid in obtaining a patch. All solutions were adjusted to a pH of 7.4, and all recordings were obtained at room temperature. Air bubbles from the pipette were removed via vibration and the pipette was placed securely into the electrode holder. The electrode consists of a Ag/ AgCl wire which connects the ion-conducting solution inside the pipette to the input of a current or voltage amplifier. In addition, a reference Ag/ AgCl electrode placed in the physiologic salt solution bathing the cells was connected to the other input of the amplifier. This helped to establish a complete electrical circuit.

Cells were viewed under a transmitted-light modulation contrast microscope (Olympus IMT-2, Tokyo, Japan) at 600X magnification (Hoffman and Gross, 1975). Cells were identified and the pipette was lowered into the perfusion chamber (Figure 3). A remote-controlled micro-manipulator device (Newport Corporation, NRC) was used to position the pipette directly over the cell. The pipette was then lowered onto the cell, and a patch was
established by closely observing resistance and current changes on an oscilloscope (Hitachi VC-6045 digital storage oscilloscope). Once this was accomplished, a slight suction was applied to the inside of the pipette to obtain a firm attachment and sealing of the membrane to the mouth of the pipette.

Detection of an acceptable seal was quantified by calculating the value of the seal resistance, which can be found from Ohm's law, where the relationship between resistance (R), voltage (V) and current (I) is as follows:

\[ V = I \times R, \quad R = \frac{V}{I} \]

Pipette resistances in these experiments initially ranged from 1.67 to 5 MΩ, which was measured between the inside of the pipette and the bath. With these experiments, the membrane voltage was clamped or held constant and picoampere ionic currents were detected through the ion channels in the patch. As the patch was being formed, the resistance increased to between 5 to 50 GΩ.

In this study, patch clamp recordings were obtained in the cell-attached configuration (Hamill et al., 1981) (Figure 4). The cell attached patch permits single ion channel recordings while maintaining the cell in an intact state. Inclusion criteria for an optimal patch were that initially, the current flow had to be equal to 0, and the baseline as seen on the
oscilloscope had to be perfectly level, without any steps at a command voltage of 20 mV. These indicated that a tight seal with an extremely large resistance had been obtained. All patch attempts that did not meet these criteria were aborted. Once a giga-Ohm seal was formed, negative pressure was applied to the cell membrane, through the pipette using a 20 cm³ syringe and monitored by a mercury manometer (Davidson, Tatakis, and Auerbach, 1990a). Pressure was applied starting at 0 cm Hg, and continuing in 1 cm increments until the patch broke.

**Signal processing**

Single-channel currents were monitored and amplified with an Axopatch 1-C patch clamp amplifier (Axon Instruments, Foster City, CA). Settings of the instrument included a -3 dB frequency of 20K, an 8-pole low-pass Bessel filter on active, a gain (α) of 0.5, a headstage gain (β) of 100, in a voltage clamp mode, with the output select set to identify current (I). The analog signal was digitized by a modified digital audio processor, PCM (Pulse Code Modulator) (Sony PCM-701ES, Tokyo, Japan) and stored on a high quality video tape using a conventional VHS video recorder. While the
analog signal was being recorded, it was passed through an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA) and displayed on the oscilloscope. Data record length depended on the activity of the channel, i.e., number of events, but typically ranged from 40 to 90 seconds at each pressure.

The data records were analyzed by taking the analog signals recorded on video tape and converting them to digital signals via the PCM, and then filtered with the Bessel filter at a cut-off frequency of 5 kHz. The analog signals were digitized at a sampling rate of 1 kHz using FETCHEX (pCLAMP) (Axon Instruments), a computer software program that retrieves the recordings from video tape and writes this information to the computer’s hard drive. The FETCHEX data files were processed using IPROC (Axon Instruments, Burlingame, CA), a program which detects ion channel events. Ion channel openings occur in discrete, identifiable clusters called “bursts”. Once a burst is detected by IPROC, it is evaluated by three criteria to determine its acceptability. First, IPROC compared the current amplitude of the signal against a window of selected current amplitudes. This window can be varied by the operator and detects valid channel openings. Second, no point during the burst can exceed 1.8 times the single channel estimated
current amplitude. Thus, multiple openings or other events which “ride” on top of the original opening level invalidate an accepted burst. The third, criterion considers the standard deviation of the putative burst from an idealized square shaped burst of the same duration and mean amplitude. If the burst does not fall within the window of acceptable standard deviations, it is invalidated. These tests for current amplitude and burst standard deviation are important in helping to determine exactly what is recognized as an ion channel and to differentiate between multiple channels. The estimated single-channel current amplitude is specified by the user and it depends on the value of the holding potential of each record. Hence, one can look at the amplitudes of several different channels and/ or the same channel at various membrane potentials.

Conductance:

The next observation was to identify what happened to the current amplitude of the ion channel as the holding potential changed. As mentioned, ion channel amplitude was derived from IPROC and can be plotted against the membrane potential (see Table III) to form an IV (current vs. voltage) curve. This curve helped to identify the conductance of each
channel and to extrapolate the reversal potential ($E_{rev}$), via a linear regression analysis (Plot-it, Scientific Programming, Haslett, MI) (Figure 5).

$P_{open}$:

The probability of a single channel being in the "open" state ($P_{open}$) was determined using NFITS (C. Lingle, Washington Univ., St. Louis, MO). Frequency histograms were created representing current amplitudes in the trace recording. These were fitted by sums of Gaussians as shown in Figure 6, where areas under the curves were calculated. The probabilities of a single channel being open ($P_{open}$) were determined from these areas under the curves using the equation: $P_{open} = 1 - P_{closed}^{1/n}$, where $n$ = the number of channels present in the patch (Table IV) and $P_{closed}$ represents the probability of the channel being in a closed state (Davidson et al., 1989; Davidson, Tatakis, and Auerbach, 1990a; Davidson, 1993). $P_{open}$ was then plotted against pressure for both control and stretch groups to identify the effect that specific pressures (in cm Hg) had on the probability of the channel being open (see Figure 7). To derive a measure of stretch-sensitivity, these points were plotted and a Boltzmann equation was fitted to the points (Davidson, 1993; Sims, Lussier, and Kracier, 1991; Sims, Kelly, and Dixon, 1991;
Statistical Analysis

The statistical analysis used in these experiments was different for the separate components in this study. First, paired t-tests were used to compare mean cell counts between subgroups in the adherence comparisons. Stretched cell numbers were compared to control cell numbers for the 1-hour, 24 hour, and 48-hour subgroups. These groups were analyzed separately, with six numbered pairs from the 1-hour group, three numbered pairs from the 24-hour group, and four numbered pairs from the 48-hour group. A significant difference would indicate that in fact many cells were lost. If this happened, it could cause a source of error in the experiment. Similarly, the converse would indicate that indeed, significant numbers of cells were not being lost as a result of stretching, and that no apparent difference existed with regard to cell number between stretched cells and controls.

Second, the mean $P_{\text{open}}$ values were compared between the different time groups for stretched cells with all other stretch cells, and between
control cells with all other controls, using an ANOVA for repeated measures. This was done to find out if it was valid to pool the data into two groups: stretched and non-stretched. The cells from each group were divided into three subgroups, of either stretched or unstretched cells: 1-hour, 24-hour, and 48-hour subgroups. For each of these three subgroups mean $P_{\text{open}}$ values were calculated at each corresponding applied negative pressure (Table V).

An identical analysis was performed to find differences between the subgroups of the control cells. Mean $P_{\text{open}}$ values of the control cells of the 1-hour, 24-hour, and 48-hour groups were compared. For these control cells, it was important to confirm that the time spent bathed in the media had no apparent effect on the ion channel activity of the cells as noted by their corresponding $P_{\text{open}}$ values.

A statistically significant finding in either of these two ANOVAs of repeated measures would identify that differences exist between the subgroups and that it would not be valid to pool the data. By the same token, the converse would indicate that no real differences exist between the subgroups and that it would be valid to pool all data into one large composite group.
Assuming that the data above could be pooled into one large composite stretch group and a composite control group, the third analysis was performed. This analysis compared the $P_{\text{open}}$ values of the two composite groups according to their applied negative pressure. Once again, an ANOVA for repeated measures was conducted with a null hypothesis that a significant difference exists between the $P_{\text{open}}$ values of the two groups due to the initial stretch of cells.
RESULTS

Cell adherence studies

The question was raised, "Are cells lost in the dishes as a result of stretching them?" To answer this question, 13 experiments were conducted to determine cell adherence between stretched cells and controls. The 13 experiments were separated into three subgroups, of which some cells were stretched and others served as controls. The first subgroup was the 1-hour subgroup, which had an n = 6. A second, 24-hour subgroup had an n = 3, and the final subgroup, the 48-hour subgroup had an n = 4. After application of stretch, the dishes were removed from the cell perturbation machine and the supernatants were removed. The cells were trypsinized, centrifuged and placed in 1 ml PBS in preparation for cell counting. The means of the cell counts for stretched and control cells were compared. Three 2-tailed paired t-tests were performed to determine if the mean cell numbers for a given stretch time differed from control cells taken at the same time intervals (Table VI). Results from these paired t-tests to detect paired differences were as follows: in the 1-hour subgroup, t(5) = 1.78 (sig. = 0.135); in the 24-hour
subgroup, \(t(2) = 1.49\) (sig. = 0.274); and in the 48-hour subgroup, \(t(3) = 3.00\)
(sig. = 0.058). Each one of these three 2-tailed paired \(t\)-tests revealed no
statistically significant differences between stretched and unstretched cell
numbers, and hence no differences between the groups. Next, the
determination of similarities between subgroups was conducted. The results
were as follows: for the 1-hour subgroup, the correlation coefficient, \(r^2 = 0.902\)
(sig. = 0.014); for the 24-hour subgroup, \(r^2 = 0.998\) (sig. = 0.038); and for the
48-hour subgroup, \(r^2 = 0.995\) (sig. = 0.005). These values indicate that a high
correlation exists between the subgroups. This also indicates that some
statistically significant similarities existed between cell numbers, when
comparing them by subgroups for duration of time.

**Metabolic Assays**

In this study, PGE\(_2\) was to be used as an identifier for cell activity, with
an attempt to tie metabolic data to electrophysiologic data. There were
numerous problems in the metabolic studies in which PGE\(_2\) was observed.
The intermittent stretching component of the cell perturbation machine
experienced frequent mechanical malfunctions. The small quantity of data
that was obtained with these experiments was found to be unreliable and inconclusive, and therefore will not be presented.

**Patch Clamping Results**

The objectives of this part of the study were to identify mechanosensitive ion channels in the G292 cells and determine whether sustained stretch influenced the stretch-sensitivity of these channels. Patch clamping was used to obtain giga-ohm seals on a total of 141 osteoblasts in the cell-attached configuration (Hamill et al., 1981). As previously observed (Davidson et al., 1989; Davidson, Tatakus, and Auerbach, 1990a; Davidson, 1993) and with KCl in the pipette, there were three distinct ion channels in these cells that could be distinguished by their individual conductances (see Figure 5 and Table III). Conductance is a term to describe the relation between current amplitude at a given electrical potential. Current amplitude was measured over a range of applied pipette potentials (-90 to 100 mV) at incremental 10 mV step intervals. Based on these recordings, the conductances of the three channels were 20 pS, 80 pS, and 200 pS for the small, intermediate, and large channels. The linear regression lines tend to
converge near -20 mV which is the resting potential \( (E_{\text{rev}}) \) of the cell, indicating that the cells were viable.

Although no attempts were made to determine the selectivity of the three different channels observed in this study, past studies have identified the selectivity of these channels in G292 cells. The literature helps to rule out the presence of \( \text{Cl}^- \) and \( \text{Na}^+ \) channels (Gofa and Davidson, 1995; Gofa and Davidson, 1993; Davidson et al., 1989; Davidson, Tatakis, and Auerbach, 1990a; Davidson, 1993), and supports the idea that these channels are in fact, \( \text{K}^+ \) selective channels of the mentioned conductances. In G292 cells, three distinct ion channels have been previously described (Davidson, Tatakis, and Auerbach, 1990; Davidson, 1993): a small, intermediate, and large ion channel (Figure 8). In this study, I elected to study the large \( \text{K}^+ \) ion channel because it is easily identifiable and has been shown to be sensitive to membrane stretch (Davidson et al., 1989; Davidson, Tatakis, and Auerbach, 1990a; Davidson, 1993).
Total cell population

When looking at the total number of cells, 95% (134/141) displayed activity of either small 20 pS, intermediate 80 pS, or large 200 pS ion channels. A great majority of the patches contained 2 different types of channels per patch, and a small percentage of the patches, 5% (7/141) lacked channel activity. Thus, it is apparent that it would have been difficult to closely observe the smaller ion channels, particularly if they were being overridden by the large channel and lost in its trace. Clearly, the largest channel was the easiest to observe. When identifying the ion channels present in the patches, 52% (73/141) of the patches contained the small 20 pS ion channel, 84% (119/141) contained the intermediate 80 pS channel, and 57% (80/141) contained the large 200 pS channel. Therefore, the intermediate 80 pS channel was the most prevalent. To subdivide the total number of 141 patches, 48 were from the one-hour subgroup, 42 were from the 24-hour subgroup, and 53 were from the 48-hour subgroup.

Single channel activity was recorded of patches where the recording medium (bath) consisted of ECS, and the pipette contained potassium-rich ICS (both mentioned previously). A typical example of the activity of the
three ion channels is illustrated in Figure 8, where three distinct channel amplitudes (small, intermediate, and large; at +40 mV) are presented in a series of traces.

Negative pressure was applied to the cells by a syringe and monitored by a mercury manometer starting at 0 cm Hg, and continuing in 1 cm increments until the patch broke (Davidson, Tatakis, and Auerbach, 1990a). This ending point was identified by a complete loss of current on the oscilloscope. The complete range of negative pressure over all cells was 0 to -10 cm Hg. Fifty six percent (79/141) of the patches endured pressure between 0 and -4 cm Hg. Twenty two percent (31/141) endured a pressure of -5 cm Hg, 13% (18/141) endured a pressure of -6 cm Hg, while 9% (13/141) underwent negative pressure of -7 to -10 cm Hg. A majority of the cells withstood negative pressure ranges from 0 to -5 cm Hg, therefore this range of pressures was used in the study which represented 78% of the total population.
**Stretched Cells**

Of the total of 141 cells, 82 were stretched and 59 were controls (Table IV). Among the stretched cells, 96% (79/82) displayed ion channel activity, and 4% (3/82) failed to display any ion channel activity as evidenced by straight line traces with only the baseline present. The patches from the 82 stretched cells were subdivided as follows: 28 were from the 1-hour subgroup, 24 were from the 24-hour subgroup, and 30 were from the 48-hour subgroup. Breaking the results down further, of the 82 stretched cells that were patched, 49% (40/82) contained the small 20 pS ion channel, 85% (70/82) included the intermediate 80 pS channel, and 56% (46/82) had the large 200 pS ion channel present. Again, the intermediate 80 pS ion channel was the most prevalent channel type in these cells.

Among the stretched cells, total applied pressure the cells could tolerate ranged from 0 to -8 cm Hg. Sixty percent (49/82) of these cells underwent pressures ranging from 0 to -4 cm Hg, 20% (16/82) of the cells remained unaltered after -5 cm Hg pressure, and 12% (10/82) were intact up to -6 cm Hg. In addition, 9% (7/82) underwent higher pressures from -7 to -8 cm Hg. As with the total population of cells, selecting the pressure range
Control Cells

A total of 59 control cells were patched. Ninety three percent (55/59) of control cells displayed ion channel activity, also with the majority of patches having two different types of ion channels. Seven percent (4/59) of these cells did not display any changes in the records or the presence of channel activity. Identification of the different ion channels present in the patch, 56% (33/59) contained the small 20 pS channel, 83% (49/59) contained the intermediate 80 pS channel, and 58% (34/59) included the large 200 pS ion channel. Once again the intermediate 80 pS channel predominated. The patches obtained from the control group were subdivided as follows: 18 were from the 1-hour subgroup, 18 were from the 24-hour subgroup, and 23 were from the 48-hour subgroup.

The complete range of negative pressure that was applied to the patches in the group of control cells was 0 to -10 cm Hg. Of these cells 51% (30/59) of the patches were intact after applied pressure from 0 to -4 cm Hg. Twenty five percent (15/59) of the patches remained unaltered after a
pressure of -5 cm Hg, 14% (8/59) were intact after -6 cm Hg of applied pressure, and 10% (6/59) underwent negative pressure of -7 to -10 cm Hg. Again the range of negative pressures selected for the study (0 to -5 cm Hg) incorporated 76% of all control cells.

"Sample" Group

From this total database of cells, all of the records were examined. Only those which could be analyzed using NFITS, and showed evidence of the large ion channel, were selected to be in the sample group. The intermediate ion channel was not chosen because it was often lost once the large channel opened. Also, when this phenomenon occurred, it was very difficult to determine $P_{\text{open}}$ values as the Gaussian curves were not distinct and it was not possible to fit curves to these Gaussians. Therefore, it was decided to use the clearest and most readily detectable channel of the three, the large ion channel. This "sample group" included a total of 33 cells, 18 of which were stretched cells and 15 were controls.

The selected cells that comprised this sample group included stretched cells, of which 33% (6/18) were from the 1-hour subgroup, 22%
(4/ 18) were from the 24-hour subgroup, and 44% (8/ 18) were from the 48-hour subgroup. Concerning the selected control cells, 13% (2/ 15) of them were from the 1-hour subgroup, 33 % (5/ 15) were from the 24-hour subgroup, and 53% (8/ 15) were from the 48-hour subgroup.

The remaining cells that were excluded from the sample totaled 98 for a variety of reasons. First, even though the records were clear and adequate, those particular records did not include the specific 200 pS ion channel that we were observing. In addition, there were some adequate patches which ruptured almost immediately thus inhibiting measurement capabilities over a range of pressures. General initial inclusion criteria included patches that when initially obtained were identified with a near perfect seal by the oscilloscope at -20 mV. This means that the two lines indicating a proper seal met in one continuous line on the oscilloscope monitor. If the patch could not meet this criteria, it was immediately aborted and a new patch was attempted. The mere fact that a good record was obtained, recorded, and followed was an indicator that the patch was acceptable. Otherwise the trace of the patch could not be followed and identified on the oscilloscope. In each patch, several quick seal test checks of -20 mV were performed to monitor the integrity of the seal present in the
patch. On several occasions, this test caused the patch to be lost.

Further inclusion criteria included records that were, clearly analyzable and where the large ion channel could be detected using NFITS at some part in the trace. These records had to be distinct, and clearly readable, with a proper seal. Less than optimal seals lead to unclear records and traces that could not be readily analyzed. Several times the baselines in these less than adequate records moved, which led to a wavy record that could not be analyzed. Subsequently, the records with these problems were not included in the study.

Can the data be pooled?

In this section, the first part of the analysis was to determine if the data obtained for stretched and control cells could be pooled into two large composite groups. Initially stretched cell data was analyzed using an ANOVA for repeated measures between the 1-hour, the 24-hour, and the 48-hour subgroups to determine if any differences existed in $P_{\text{open}}$ between the subgroups. This analysis was chosen to determine if any differences existed in $P_{\text{open}}$ values between the groups over a series of negative applied
pressures (the repeated measure). Initially, the $P_{\text{open}}$ values from the stretch group were observed at each individual pressure (see Table IV - top half), keeping the subgroups separate with respect to number of hours of initial stretch (type). The result of this ANOVA for repeated measures among stretch subgroups was $F(2,17) = .22, p = .813$. This indicates that no significant difference existed in $P_{\text{open}}$ values between any of the stretch subgroups at each individual level of applied pressure. Subsequently, the $P_{\text{open}}$ values from all three stretch subgroups were compared over the range of applied negative pressures. Again, an ANOVA for repeated measures was conducted to look for differences where $F(5,17) = .55, p = .838$. This finding confirmed that these stretch subgroups were not different, even over the entire range of applied negative pressures, but were in fact, all similar. From these two findings, it was concluded that it was valid to pool the 1-hour, the 24-hour, and the 48-hour stretch subgroups, as they were not found to be significantly different, and consider them as one large stretched cell group.

Similarly, the same procedure was taken to see if control cells could be pooled together into one large control group. Control cell data were compared using an ANOVA for repeated measures between the 1-hour, the 24-hour, and the 48-hour control subgroups to determine if there were any
differences in $P_{\text{open}}$ values that existed between these subgroups (see Table IV - bottom half). Then, similar to the stretched cell data, the $P_{\text{open}}$ values were examined between the subgroups of control cells taken at 1-hour, 24-hours, and 48-hours at each individual pressure using an ANOVA for repeated measures, where $F(2,14) = .14$, $p = .875$. This indicated that no significant difference existed in $P_{\text{open}}$ values between any of the control subgroups at each level of applied negative pressure. Subsequently, the $P_{\text{open}}$ values for all three control subgroups were compared over the range of all applied negative pressures for any differences. Similarly an ANOVA for repeated measures was conducted, where $F(5,14) = 1.12$, $p = .384$. Likewise, this test revealed no statistically significant differences between any of the groups over the entire range of applied negative pressures. Therefore, it was concluded that the control subgroups were in essence similar, and it was valid to pool the 1-hour, the 24-hour, and the 48-hour control subgroups, and consider them as one large control cell group.
Control vs. stretch groups

The data for each mean $P_{\text{open}}$ value with corresponding pressure is shown in Table VII. The stretch group is represented in Figure 7 by the upper fit curve, which was derived from plotting the mean $P_{\text{open}}$ values at corresponding pressures. As mentioned, the plotted points were fit by Boltzmann equations (see Davidson, Tatakis and Auerbach, 1990; Davidson, 1993) and have ranges for the standard error of the mean, as represented by the vertical bars at each pressure point. The same calculations were performed for the pooled control cells, which are also shown in Table VII. Many interesting findings were derived from these results.

Initially, the curves for both groups of cells look similar from 0 to -3 cm Hg of applied negative pressure, where the plotted points on the graph (Figure 7) are very close to each other. This is also the case for the ranges of the SE means, as they overlap over this range of pressure. Then significant differences occur between -4 and -5 cm Hg pressure. At -4 cm Hg of pressure, the mean $P_{\text{open}}$ value for the stretch group jumps to nearly 0.4, while the control group has a mean $P_{\text{open}}$ value of nearly 0.15. The SE means are also very different and do not intersect, indicating that there is a
big difference between the two groups at this point. The same is true at
-5 cm Hg of pressure for both groups, where for the stretched cells, mean
$P_{\text{open}}$ approaches 0.5, while for the controls, mean $P_{\text{open}}$ declines to below
0.1. Again, the SE mean ranges are very different and do not overlap,
indicating large differences between the two groups at this pressure.

The next step was to compare these two pooled stretch and control
groups to each other to look for statistically significant differences. Again an
ANOVA for repeated measures was used, where mean $P_{\text{open}}$ values were
compared with corresponding applied negative pressure for both groups to
test the main effects that stretch had on the cells. The test compared the
difference between stretched cells and controls with respect to their $P_{\text{open}}$
values was found to have a $F(1,14) = 6.04$, and $p = .028$. A significant
difference in the responses was observed between the two groups with
regard to stretch perturbation. This implies that a true change in the
responsiveness of the large 200 pS ion channel takes place when the cell is
stretched. When the $P_{\text{open}}$ values of the two groups were observed across
pressure, again a significant difference was observed, $F(5,70) = 4.54$ and
$p = .001$. This demonstrates that the two groups are quite different when
observed over the range of applied pressures. The mean $P_{\text{open}}$ values from
this ANOVA of repeated measures is displayed in Table VII.

In this study, two dependent variables were measured: channel current amplitude and $P_{\text{open}}$. From these measures, conductance, maximum $P_{\text{open}}$, stretch sensitivity, and the pressure at 1/2 the maximum $P_{\text{open}}$ values were derived.

First, conductance was determined by taking the ion channel amplitudes for current (in pA) (Table III) and plotting the change in amplitude along the range of applied voltage. This relationship is equivalent to the slope of the linear regression which was fit for currents plotted over the range of electrical potentials (Figure 5). These slopes represent the conductances for the three ion channels that were identified in this study, and are described in pico Siemens (pS), which is the inverse of the resistance ($1/R$, which equals $I/V$ from the equation $V = I \times R$). The conductances for the small, intermediate and large channels were found to be approximately 20 pS, 80 pS, and 200 pS respectively. The regression lines of all three channels converged at around -30 mV to -40 mV, which is the reversal potential of the cell ($E_{\text{rev}}$).

Next, the maximum value for $P_{\text{open}}$ was found for both groups (Table VIII). This value represents a plateau or a maximum threshold for ion
channel activity in the two groups. This is represented by the highest points found on the curve in Figure 7, which were taken from measured mean $P_{\text{open}}$ values in Table VII at corresponding pressures. Ideally, maximum $P_{\text{open}}$ would equal "1", indicating the channel being open 100% of the time without closing. Here, this is obviously not the case, but does differ between the stretch and control groups. The $P_{\text{open}}$ max = 0.489, which was attained at a corresponding pressure of -5 cm Hg for the stretch group. The $P_{\text{open}}$ max = 0.16 for the control group, which was attained at a corresponding pressure of -4 cm Hg. Hence a marked difference in channel activity exists between the two groups.

Stretch sensitivity was derived directly from a Boltzman equation, which was used to fit the sigmoid relationship that exists between the $P_{\text{open}}$ values to the corresponding negative pressure applied (Figure 7). The unknown value ($\theta$) in this equation is the sensitivity to pressure or the stretch sensitivity. $\theta$ is an intrinsic property of the ion channel and is a single measure for all the $P_{\text{open}}$ values in the sigmoid curve. The stretch sensitivities were 0.22 for the stretched cells and 0.07 for the controls (Table VIII). This indicates that the stretched cells are more than three times more responsive to the stretching conditions than were the controls. The tendency for $P_{\text{open}}$ to
increase or decrease is reflected by $\theta$, which reveals changes in the kinetic properties of the ion channels. The rate of change of $P_{\text{open}}$ is also dependent upon the difference in $\theta$, which is represented in Figure 7 by the dissimilar shapes that exist when comparing the stretch and control curves.

Finally, the pressure at $1/2 \ P_{\text{open}}$ max was derived indirectly using the Boltzmann equations (Figure 7). The fitted values of each curve were observed and $P_{\text{open}}$ values were determined at $1/2$ the maximum value for both stretched cells and controls. The negative applied pressure was determined from $1/2 \ P_{\text{open}}$ max values for both groups, using the Boltzmann equation. The corresponding pressures at $1/2 \ P_{\text{open}}$ max were as follows: -2.96 cm Hg for the stretch group and -1.32 for the controls (Table VIII).

Interpreted, this means that the stretch group reached $1/2$ of the maximum channel opening potential at nearly -3 cm Hg of applied negative pressure, where controls reached this same level of maximum channel opening at -1.3 cm Hg of pressure. It is also apparent that the control cells reach this "half-way point" much earlier than do the stretched cells. Again, these apparent differences must be associated to the initial strain applied to the cells, because all other succeeding parts of the experiment were kept under identical conditions.
The results from the metabolic studies in which PGE$_2$ was observed were unreliable and inconclusive. Originally PGE$_2$ was to be used as an identifier for cell activity, with an attempt to tie metabolic data to electrophysiologic data. Due to multiple malfunctions of the stretch apparatus applying an intermittent stretch to the cells, it was determined that this data would not be presented.
DISCUSSION

The reason for selecting the G292 cell line

In the present study, human G292 osteoblast-like osteosarcoma cells were selected as a model for testing the effect of mechanical perturbation on the activity of ion channels. In the past, one drawback of using osteoblastic tissue culture has been the heterogeneity and difficulty in isolating a single cell type (Nijweide, Van der Plas and Scherft 1981; Davidson, 1993). This presents a problem with regard to uniformity and comparison between responses obtained in identical osteoblastic cells. In this study, G292 cells were chosen because of their availability, homogeneity, and osteoblast-like characteristics (Peebles, Trisch, and Papageorge, 1978; Shupnik and Tashjian; 1982).

In contrast to using homogenous, well defined cells for in vitro studies, experiments using primary cultures are much more difficult to perform. First, if primary cultures are used, the researcher needs to deal with problems of heterogeneity or a difference of cell types and cell functions. Next, the possibility of cell to cell interactions may be present, which may distort the view of individual cell response to application of stress. Finally, specific
hormones found circulating in vivo may inhibit or accelerate the levels of cell metabolites and/or ion channel activity.

G292 cells are derived from human cells and have a population of resident ion channels that have been well characterized (Davidson, Tatakis, and Auerbach, 1990; Davidson, 1993). In these cells, mechanosensitive, K⁺-selective, and 50 pS arachidonic acid-sensitive ion channels have been detected (Davidson et al., 1989 & 1990b; Davidson, Tatakis and Auerbach, 1990a; Davidson, 1993).

**Mechanical stimulation**

The purpose of the experiments described in this thesis was to identify the cellular changes that take place when osteoblast-like cells are subjected to stretch in vitro. The present study was designed to simulate the strain that is present in surrounding bone when physiologic, light forces are used during ideal orthodontic tooth movement, and to identify what events occur at the cell membrane during this physiologic condition. Presently, there have not been any single ion channel studies of osteoblast cells which could be associated with the forces applied to bone during orthodontic tooth movement.
In this experiment, G292 cells were cultured, separated into groups, then stimulated mechanically using the device described in detail in Materials and Methods (Norton et al., 1995; Norton et al., 1992, Andersen and Norton, 1991; Andersen, Pedersen, and Melsen, 1991). Many other studies have applied various kinds of mechanical forces to bone or cartilage cells in culture. In a manner similar to the one in this study, the stretching force was applied by deformation of the culture dish (Hasegawa et al., 1985). Other studies have applied tensile force on collagen ribbons (Yeh and Rodan, 1984), hydrostatic compressive force (Rodan et al., 1975b; Klein-Nulend et al., 1987), and centrifugal force (Duke, 1983; Inoue et al., 1993) as mechanisms of transferring mechanical stress to cells.

Cell adherence

An important part of this experiment was to determine if cells were being lost from the bottom of the flexible dishes. This point is important because if cells were lost while being stretched, it could have led to data from which faulty interpretations might have been extracted. Several studies have found that stretching bone cells leads to increases in cell number (Berger & Veldhuijzen, 1993; Raab et al., 1991; Rubin & Lanyon 1984; Rubin &
The results from 13 independent experiments in this study seem to indicate that cell numbers seem to remain constant regardless of if they were stretched or not. When cells were separated into subgroups of 1, 24, and 48 hours of stretch and compared by paired t-tests, the paired differences for these subgroups had a 2-tail significance of 0.135, 0.274, and 0.058 respectively. These findings help to give confidence that a significant loss of cells did not occur throughout these or subsequent experiments of the overall study. There is, however, a remote possibility that cells were being lost, but the cells were proliferating as found in other studies. This proliferation rate could be similar for both modalities, resulting in no net loss of cells. Although this is possible, I feel that it is unlikely because one would not expect an enormous proliferation of most cells within 48 hours. It would also be highly unlikely that this phenomenon would occur equally in all 13 experiments.

The original intention was to conduct the perturbation at different time intervals. However, I ran into numerous problems with breakdown and malfunction of the cycling aspect of the apparatus, and had to make changes from my original plans. Subsequently, I elected to stretch the cells continuously, thus mimicking a constantly applied orthodontic force. The
cells were separated into three distinct groups, and then prepared for the electrophysiology studies. The cells were divided into the following groups: 1) perturbation at the start of the experiment lasting for a duration of one hour of applied continuous stretch, 2) a second group receiving 24 hours of continuous stretch, and 3) a final group receiving 48 hours of continuous stretch. These groups were the same for both control and experimental cells. The selected control group did not undergo mechanical perturbation, but was subjected to the same conditions in the incubator, feeding, seeding as the experimental cells. Subsequently, the patch clamp recording technique (Hamill et al., 1981) was applied to identify resident ion channels and analyze their properties, which may have participated in these events. Cell-attached patch conditions were applied to investigate single-channel currents, which were then compared to each other.

PGE$_2$ production in response to stretch

In this study, it was also proposed to observe changes in levels of PGE$_2$ as an indicator for the response of bone to stretch. This too posed a problem as quantification of levels of prostaglandin was difficult, due to an absence of a clear association with applied stretch. In the early stages of
development of the experiment, cells were intermittently stretched for up to a period of 72 hours. After running these intermittent stretch tests, prostaglandin levels were measured from the remaining supernatant. Results from this portion of the experiment reported no consistent change in levels of prostaglandin production. This suggests that prostaglandin production may not be related to mechanical stretch of these particular osteoblastic cells. This is consistent with the findings reported by Sandy (1989), but oppose many findings previously reported, where osteoblasts showed increased levels of PGE$_2$ in response to stretch (Yeh and Rodan, 1984; Harrell et al., 1977; Murray and Rushton, 1990; Reich et al., 1990; Reich and Fangos, 1991; Reich and Fangos, 1993). Several laboratories have reported that physical strains were transduced into a chemical signal through production of prostaglandins, which led to intracellular cAMP production, and subsequently to stimulation of DNA synthesis of bone cells (Binderman et al., 1984; Yeh and Rodan, 1984; Hakeda et al., 1985; Norrdin et al., 1990; Raisz and Fall, 1990). Possible reasons for this variance in findings could be due to different phenotypic expressions in cell lines. It is also probable that the G292 cell line does not respond to mechanical stimulation by producing PGE$_2$. Also the technique for detection of
prostaglandin levels is much more sophisticated in this study because it can test for all types of prostaglandin at precise measurements. At present, it is the state of the art method for accurate prostaglandin detection and measurement. Previous findings used less accurate methods to quantify prostaglandin levels. Further research needs to be carried out in this area using a more sophisticated method for prostaglandin analysis, as outlined in this study.

Mechanosensitive ion channels

Mechanically sensitive channels have been characterized in a wide variety of cells from different tissue types (Sachs, 1988; Morris, 1990). Others have characterized osteoblast-like osteosarcoma cell lines UMR-106.01 (Duncan and Misler, 1989), MG-63 (Davidson, et al., 1990b), and G292 (Davidson, 1993). Also, mechanically induced whole cell and single channel current activation with associated mechanosensitive channel activity has been found in yeast spheroplasts (Gustin, 1991), fungal cells (Zhou et al., 1991), mammalian smooth muscle (Davis, Donovitz, and Hood, 1992), rat cardiac muscle (Sadoshima et al., 1992; Kim, 1993), chick heart muscle (Ruknudin et al., 1993), *Lymnaea* (snail) neurons (Small and Morris, 1994),
and rabbit airway epithelial cells (Kim et al., 1993). There is an overall general assumption that mechanosensitive ion channels are gated by changes in membrane tension, rather than by pressure itself. It is still unclear whether the gating tension is exerted by the underlying cytoskeleton (Guharay and Sachs, 1984) or by the lipid bilayer (Martinac et al., 1990). This pathway still needs to be elucidated.

Recently, one group applied chronic, intermittent strain on the mechanosensitive cation channels in UMR-106.01 osteoblast-like osteosarcoma cells (Duncan and Hruska, 1994), using a Flexcell apparatus. This apparatus applied a non-uniform strain pattern to the cells, ranging from 120,000 μE at the edge of the well to 0 μE at the center (Banes et al., 1990). Observations of these effects were made using patch clamp techniques (Duncan and Hruska, 1994). They found that strained cells demonstrated significantly larger increases in whole cell conductance when subjected to additional mechanical strain, in comparison to non-strain controls. These findings indicate that intermittent strain led to a reduction in activation threshold of the stretch activated cation channel when compared to control cells. This is similar to the findings in this study where a continuous strain was applied, revealing a marked increase in ion channel activity with
stretched cells ($P_{\text{open}}$ max = .489 for the stretched cells and $P_{\text{open}}$ max = .159 for the controls). The hypothesis from this study is that the stretch-activated cation channel acts as a mechanotransducer for the activation of bone remodeling via mechanical strain.

In another study looking at *Xenopus* oocytes, mechanosensitive channels were found to open transiently in response to the suction that was applied to the membrane patch in step changes (Hamill and McBride, 1992). This adaptive behavior was thought to occur because of a reduction in $P_{\text{open}}$ rather than a change in the conductance of the channel. They found that channel adaptation was highly voltage dependent, where it was most apparent at resting or hyperpolarized potentials, but was absent at strongly positive potentials. They thought that mechanosensitive channel activity was due to different stages of the membrane-cytoskeleton decoupling, caused by increased mechanical stress as greater suction was applied with a corresponding increased $P_{\text{open}}$. This study, however, used suction for both main groups of cells. The only variable that was different was the initial applied stretch that the cells underwent prior to electrophysiologic studies. Even though both groups had negative pressure applied to their cell membranes, they would be expected give similar results in $P_{\text{open}}$ if initial
stretch did not matter. Overall, a real difference exists which seems to be related to stretching the cells prior to patch clamping.

Findings

The findings from this study helped in understanding that cell adherence was not significantly disturbed and that stretching cells does not lead to a significant cell loss in this system, with these cells. This was important to provide confidence that all experiments were identical with regard to cell number. If a variance or deviation in cell number occurred following stretching, it would have been extremely difficult to interpret the data and put in question the validity of the experiments.

The results of these experiments show that a significant difference exists with regard to their $P_{\text{open}}$ state, when these G292 osteoblast-like cells are stretched in vitro. These differences are apparent when comparing stretched cells to unstretched or control cells. This is an important finding because it implies two main points. First, that these channels do indeed respond to stretch activation, indicating that these are mechanosensitive ion channels. This is consistent with previous findings (Guharay and Sachs, 1984) which gave an operational definition for identifying stretch activated
channels. They mentioned that $P_{\text{open}}$ must increase with membrane stretch. This is exactly what was observed in this study with both control and experimental groups. Second, it was found that the initial period of stretch does have a pronounced effect on the properties of the ion channel. It was thought that the time interval from dish removal from the stretch machine until obtaining the patch could lead to a "cell relaxation" and show a decrease in responsiveness. Although this is possible, when comparing the mean values for the time it takes before the patch is obtained, these times are practically identical for stretch and control cells (85 mins. and 84 mins. respectively - see Table IV). This might be determined more accurately if obtaining adequate patches was not so difficult and took so much time. In an ideal experimental setting, all patches could be obtained immediately, thus undergoing no relaxation time. At present, it is unpredictable as to how much data one would obtain on a given day of patch clamping.

Third, the interval of time that the channel is open was found to be related to the variable of stretch when stretched cells were compared with controls. Stretched cells reach a probability of being open ($P_{\text{open}}$) approximately 2 1/2 times more than do control cells. This strongly suggests that initial stretch has a marked effect on the ion channel activity in
osteoblastic cells. The fact that these ion channels respond to stretch in this manner also helps to identify them as being stretch activated channels. Continued investigation in this area would be useful, especially if a large study could be conducted with a sufficient sample size to corroborate the findings obtained here. In addition, other investigations could make similar observations and comparisons by looking at the intermediate and the small ion channel and identifying their activity over the same series of applied negative pressures. This study did not lend itself to these last observations due to the time needed to identify these parameters for the large ion channel alone.

**Summary**

In summary, three types of resident ion channels were identified and quantified according to their conductances. It was also found that stretching osteoblastic cells led to a large and significant increase in the time that an individual ion channel remains open or active. Electrophysiological studies confirmed that cells which underwent stretch responded quite differently when compared to controls. The cells attained different maximum $P_{\text{open}}$ values, where the channel activity plateaus: .489 for the stretched cells and
.159 for the control cells. Also, the stretch cells were found to reach their 1/2 maximum $P_{\text{open}}$ value more quickly than did the controls. Stretched cells were found to be three times more stretch sensitive than controls. The results of this study could be interpreted as an overall increase in sensitivity to stretch, which is contrasted to a marked relative inactivity in resting osteoblasts (controls). This may tell the clinician that, indeed, bone cells behave differently when subjected to stretch conditions, as found when teeth are moved with orthodontic forces, than they behave under resting conditions.

The data obtained in this study helps to identify the single ion channel activity found in these osteoblastic cells. Furthermore, this activity helps to corroborate the concept that these cells have special receptors that are particularly sensitive to stretch. These subsequently turn on a cascade of intracellular mechanisms which lead to the observed physiologic changes, as discussed previously. It is difficult to draw clinical conclusions based on membrane cell biology, but this link may be made with further investigation in the near future.
Table I: Raw cell adherence values. This table displays the raw data from the cell adherence portion of the study. The right column identifies the number of the experiment (13 experiments in total). The next two columns, "Type" and "N" identify which type of cell and the number of dishes that were tested in that particular experiment. The last three columns give mean cell counts (Mean Counts), the standard deviations (St. deviation), and standard errors of the mean (SE of mean) for each group of cells, for each experiment. The asterisks under the control group in experiment 9 indicate that no standard deviation or standard error of the mean was available. This is because n = 1 for that group, and therefore no comparisons could be made to determine these missing values.
Raw Cell Adherence Values

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Table I
Table II: Paired differences of cell adherence for stretch and control groups. In this table, the 13 experiments were divided into 3 subgroups according to the amount of time they underwent stretch (1-hour, 24-hour, and 48-hour subgroups). This is shown in the first column. The second column, "No." indicates the number of cell adherence experiments that were performed for the three corresponding subgroups. Next, mean differences were calculated with corresponding standard deviation (St. deviation) and standard error of the mean (SE of mean). Finally, the t-values, the degrees of freedom (df), and two tailed significance (2-tail Sig.) were calculated for each of the three subgroups. No statistically significant differences were found between stretch and control groups from each subgroup.
Paired Differences of Cell Adherence for Stretch and Control Groups

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<th>Time</th>
<th>No.</th>
<th>Mean</th>
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<th>t-value</th>
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<th>2-tail Sig.</th>
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<td>88651.295</td>
<td>44325.647</td>
<td>3</td>
<td>3</td>
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</table>

Table II
Table III: IV curve values. This table displays 21 series of current amplitude changes over varying electrical potential changes for the three types of ion channels found in G292 cells. The left most column displays the voltage change which ranged from -90 mV to 100 mV. These electrical potential changes occurred in increments of 10 mV. The numbers in the top row identify the experiment from which these recordings were made. The values between these headings represent the measured mean current amplitude for that segment of the record. Below this matrix of values, is a row which identifies in simple terms, which type of ion channel was present (S = small 20 pS channel, $l =$ intermediate 80 pS channel, and $L =$ large 200 pS channel). The total number of identified channels are listed below this according to channel type. Finally at the bottom, the average amplitude for each type of channel was calculated. Here, the values show what the mean current amplitudes would be at an electrical potential of 40 mV. This voltage was chosen because it was used extensively throughout the study.
Table IV: Mean $P_{\text{open}}$ Values. This table identifies the calculated $P_{\text{open}}$ values for each cell at the corresponding applied negative pressure (in cm Hg). The upper half of the table shows the raw data obtained for the stretched cells, and the lower half displays the data for the control cells. The column marked “File” identifies which starting trace the record was taken from. The “Cell” column represents the number of the cell that was actually patched on that particular recording date. Next, the numbers in the “Hrs” column tell which subgroup the successfully patched cell belonged to (i.e. 1, 24, or 48 hour group). The next column, “St. time” identifies how many hours that cell was stretched (i.e. 0, 1, 24, or 48 hours; 0 hours signified control cells). “TBP” indicates the time that it took when removing the cells from the incubator to when the patch was actually obtained. The rest of the columns represent the $P_{\text{open}}$ values at the corresponding amount of applied negative pressure. This pressure was applied in increments of -1 cm Hg. The column “$P_{\text{op} \ 0}$” represents the $P_{\text{open}}$ values as the patch was initially formed with no applied negative pressure. The subsequent column, “$P_{\text{op} \ -1}$” gives the $P_{\text{open}}$ values when -1 cm Hg of pressure is applied. This continues with all cells until the patch broke which is represented by the empty boxes. A value of “0” indicates that only the baseline (no channel activity) was detected. All values
are below "1" which if present would indicate that the channel was
continuously open without being in the closed state (theoretical). In addition,
the totals in bold type below each main group give from left to right: the total
number of patched cells in the group and the mean time it took before the
patch was obtained. The next series of numbers is the mean $P_{\text{open}}$ value for
each corresponding step of applied negative pressure. When the patch
ceased, the mean $P_{\text{open}}$ values at subsequent pressures only included those
cells that were able to hold up under that pressure.
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</tr>
</tbody>
</table>

Table IV
Table V: Patch clamp data for all cells. This table identifies the types and numbers of all patched cells obtained in this study and from which subgroup they belong. Numbers of stretched and control cells were totaled and placed in their corresponding subgroup (sg) (i.e. 1-hour sg, 24-hour sg, and 48-hour sg). In addition, the percentages alongside the numbers represent that portion of the total numbers of cells for that particular group. Below, the totals were added according to each subgroup, with total numbers being present at the far right of the table.
Patch Clamp Data for All Cells

<table>
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<tr>
<th></th>
<th>1 hr group</th>
<th>%</th>
<th>24 hr group</th>
<th>%</th>
<th>48 hr group</th>
<th>%</th>
<th>total</th>
</tr>
</thead>
<tbody>
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<td>37</td>
<td>82</td>
</tr>
<tr>
<td>control</td>
<td>18</td>
<td>31</td>
<td>18</td>
<td>31</td>
<td>23</td>
<td>39</td>
<td>59</td>
</tr>
<tr>
<td>Totals</td>
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<td>33</td>
<td>42</td>
<td>30</td>
<td>53</td>
<td>38</td>
<td>141</td>
</tr>
</tbody>
</table>

Table V
Table VI: Patch clamp data for the "sample" group. This table identifies the "chosen" sample group. This sample was selected strictly on the ability to clearly read and analyze those records which contained the large ion channel. Similar to Table V, stretch and control groups were divided into subgroups with the additive totals and corresponding percentages below and to the right for each subgroup. Composite totals are represented at the far right.
Patch Clamp Data for the "Sample" Group

<table>
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<tr>
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<th>%</th>
<th>24 hr group</th>
<th>%</th>
<th>48 hr group</th>
<th>%</th>
<th>total</th>
</tr>
</thead>
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<tr>
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<td>33</td>
<td>4</td>
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<tr>
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<td>13</td>
<td>5</td>
<td>33</td>
<td>8</td>
<td>53</td>
<td>15</td>
</tr>
</tbody>
</table>

Table VI
Table VII: Mean $P_{\text{open}}$ data for the "sample" group. This table gives the mean $P_{\text{open}}$ values for each corresponding pressure for the sample composite stretch and composite control groups. The right most column represents the pressure applied in cm Hg. The next column "n" identifies the number of patched cells that remained intact at that corresponding pressure. The column with the heading "mean" identifies the mean $P_{\text{open}}$ values for that corresponding pressure. The next two columns give the analogous standard deviations (St. deviation) and standard errors of the mean (SE mean).
Mean $P_{\text{open}}$ Data for the "Sample" Group

<table>
<thead>
<tr>
<th>s-stretch</th>
<th>pressure</th>
<th>n</th>
<th>mean</th>
<th>St deviation</th>
<th>SE mean</th>
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<tr>
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<td>-5</td>
<td>8</td>
<td>0.489</td>
<td>0.427</td>
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</table>

<table>
<thead>
<tr>
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<th>n</th>
<th>mean</th>
<th>St deviation</th>
<th>SE mean</th>
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Table VII
Table VIII: Findings from the Boltzman equation. This table corresponds with Figure 7, the Boltzman equation of experimental findings. Here, composite stretch and control groups were compared for their maximum $P_{\text{open}}$ values ($P_{\text{open max}}$), the pressure that corresponds to 1/2 the maximum $P_{\text{open}}$ value ($P_{\text{open 1/2}}$), and stretch sensitivity. $P_{\text{open max}}$ represents the maximum probability of the ion channel to be in an open state and tends to plateau at this maximum value. $P_{\text{open 1/2}}$ identifies the negative pressure at which the composite group reaches 1/2 of its maximum value. This measurement was chosen to help identify how much applied negative pressure it takes to reach this 1/2 maximum $P_{\text{open}}$ values. Stretch sensitivity identifies how responsive the channels are to opening. Here, the stretched cells were more than three times more responsive to opening when compared to controls.
Findings from the Boltzmann equation

<table>
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<th>Popen 1/2 Max</th>
<th>S Sensitivity</th>
</tr>
</thead>
<tbody>
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<td>Controls</td>
<td>0.16</td>
<td>-1.32</td>
<td>0.07</td>
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</table>

Table VIII
Figure 1: Cell perturbation machine. This machine is piston driven (3) with connecting arms (2) to the upper table (1). It is possible to place up to 12 dishes concurrently (4) and adhere them to the upper table with wax. When the piston is down (A) the dishes are stretched on their flexible bottoms over the precision ground domes resting on the lower table. As the piston rises (B), the dishes are lifted away from contact of the domes below. Due to malfunction of the piston, a continuous stretch (A and not B) was applied for 1, 24, and 48 hours, mimicking ideal orthodontic tooth movement.
Cell perturbation machine

Figure 1
Figure 2: Strain calculation for watchglass fabrication. This series of formulas were established to help construct the watchglasses (B) so that they would help to deliver a 1% strain (ε) to the flexible bottoms of the Petriperm® culture dishes (A) as they are stretched over the watchglass (B). The explanation of the symbols are as follows:

- R - represents the radius of the circle defined by the watchglass
- α - identifies the angle represented from the edge of the watchglass to its midpoint within the circle of radius R
- a - is the distance from the center of the circle of radius R in which the watchglass lies, to the bottom of the flexible bottom dish
- h - is the vertical height of the watchglass
- l - is the arc length of one half of the watchglass from the bottom to its midpoint
- r - represents one half the distance from the edge of the dish to its center
Strain calculation for watchglass fabrication

\[
\sin \alpha = \frac{r}{R}, \quad \alpha = \arcsin \frac{r}{R}
\]

\[
l = \alpha R = (\arcsin \frac{r}{R})R = R\left(\frac{r}{R}\right) + \left(\frac{r}{R}\right)^3 + \frac{3}{40}\left(\frac{r}{R}\right)^4 + \ldots
\]

\[
\frac{l}{r} - 1 = \frac{1}{6}\left(\frac{r}{R}\right)^2 + \frac{3}{40}\left(\frac{r}{R}\right)^4 + \ldots
\]

\[
\frac{l-r}{r} = \varepsilon = \frac{1}{6}\left(\frac{r}{R}\right)^2 + \frac{3}{40}\left(\frac{r}{R}\right)^4 + \ldots
\]

Rearranging and only including the main factors

\[
\varepsilon = \frac{1}{6}\left(\frac{r}{R}\right)^2 \quad \text{or} \quad R^2 = \frac{r^2}{6 \varepsilon}, \quad R = \frac{r}{\sqrt{6 \varepsilon}}
\]

Figure 2
**Figure 3:** Schematic of the patch clamping technique. The micropipette is lowered into the bath chamber which rests on a transmitted-light modulation contrast microscope. A “patch” is obtained where a small portion of the cell membrane forms a seal with the tip of the pipette. This attempt is made to capture a single ion channel within the borders of the pipette from which recordings can be made. A syringe is connected to the pipette to apply a controlled amount of negative pressure. This pressure is measured by a mercury filled manometer, which is also connected to the syringe and to the pipette. Readings were taken in cm of Hg pressure. Pressure is applied and channel activity recorded until the patch breaks or becomes distorted.
Figure 3

Schematic of the patch clamping technique
Figure 4: Cell attached patch and excised patch configurations. This figure is an addition to the schematic in Figure 3. As a “patch” is made, this configuration is said to be a cell attached patch (a). Ion channel recordings can be performed in this state, as was done in this study, or the operator can elect to tear the piece of membrane from the cell and form an excised patch (b). This was also performed by accident on one of the recordings in this study. An alternate form, called a whole cell patch (c) can be made from the cell attached patch (a) by eliminating that portion of membrane bounded by the pipette tip. This type of recording helps identify many channels simultaneously (records the cell as a whole) but was not used for this study.
Cell attached patch and excised patch configurations

Figure 4
**Figure 5**: Conductance graph (IV graph). This graph represents the current/voltage relationship for the three types of ion channels identified in this study for G292 cells. Recordings were taken of the ion channels with changes in voltage. The current amplitudes of the channels varied in a linear relationship according to changes in electrical potential as described by the formula \( pA = \text{slope} \times \text{mV} + \text{intercept} \). This relationship is derived from the basic formula, \( y = m \times x + b \), where \( y \) is the amplitude of the current in pA and \( x \) is the electrical potential in mV. Here, the circles & solid dashed lines represent the small channel, which is approximately 20 pS. The squares & lightly dashed line represent the intermediate channel, which is approximately 80 pS. Finally, the triangles & solid line represent the large channel, which is approximately 200 pS. This was the channel selected for observation in this study. The approximate point or area where all three lines converge represents the reversal potential \( (E_{rev}) \). The solid shapes represent specific data points from the study as shown in Table III. The lines connecting these shapes represent regression lines to best fit the corresponding data points.
Conductance graph (IV graph)

pA=[Slope] * mV + intercept

Figure 5
Figure 6: NFITS frequency histograms. These frequency histograms come from the NFITS computer program (C. Lingle, Washington Univ., St. Louis, MO) and plot amplitudes of the channels identified in the patch clamp recordings. Figures 6A and 6B collectively display a progression of frequency histograms and traces from a typical cell from this study, at 40 mV as negative pressure is applied. The histograms are fit to Gaussian curves where the areas under these curves are calculated and placed in the formula: 
\[ P_{\text{open}} = 1 - P_{\text{closed}}^{1/n} \]. This helps to determine the probability of a single ion channel being in an open state.

Figure 6A: The top graph and trace identify the cell just after the patch had been made at 0 cm Hg of pressure. The only curve present is for the baseline or a current amplitude of 0 pA. The trace below the histogram displays virtually no channel activity, hence one peak is present in the histogram. The trace was essentially identical for 0, -1, -2, and -3 cm Hg of pressure applied to the cell, and are not included here to avoid repetition.
NFITS frequency histograms

Figure 6A
**Figure 6B:** This is a continuation of the record from the same cell at -4 and -6 cm Hg of applied pressure. The second frequency histogram (at the top of the page) represents the recording at -4 cm Hg of pressure, and has two peaks. The left most or first peak and the area under this peak correspond again to the baseline or 0 pA current amplitude recorded on the trace. The peak farthest to the right or third peak represents the 8.5 pA or large channel. Again, areas under these curves are calculated and applied to the formula: $P_{\text{open}} = 1 - P_{\text{closed}}^{1/n}$ to find the probability of a single ion channel being open. The trace below this top frequency histogram helps to visualize the activity of the channel at -4 cm Hg of pressure. Here, the lower line is the baseline or closed state of the ion channel, while the upper line represents the ion channel in the open state.

The lower histogram represents the channel recording at -6 cm Hg pressure. In this frequency histogram, two peaks are present. The left peak and area correspond to the baseline or the 0 pA current amplitude. The right peak and area identify the 8.5 pA or large ion channel. The corresponding trace is shown below the histograms. Here, as more negative pressure was applied, the channel showed a tendency to increase in activity. This is evidenced by observing all of the previous traces and the frequencies of the
histograms and comparing them to the particular negative pressure that was applied at that point in the record.
NFITS frequency histograms

Figure 6B
Figure 7: Boltzmann equation of experimental findings ($P_{\text{open}}$ vs. pressure). This graph displays the change in $P_{\text{open}}$ with respect to cm Hg of applied negative pressure. The open circles represent the mean $P_{\text{open}}$ values for the stretched cells and the open squares represent the mean $P_{\text{open}}$ values for the control cells. Generally, the $P_{\text{open}}$ values are the same for 0, -1, -2, and -3 cm Hg of pressure. Significant differences are present between the two groups for values of -4 and -5 cm Hg of pressure. Stretched cells reached a maximum $P_{\text{open}}$ value of .489 while control cells reached a maximum $P_{\text{open}}$ of only .159. A Boltzmann equation (see Davidson, Tatakos and Auerbach, 1990; Davidson, 1993) was fitted for each of these curves (represented by the dashed lines).

From this graph, 1/2 maximum $P_{\text{open}}$ value and stretch sensitivities can be calculated. 1/2 maximum $P_{\text{open}}$ values were reached at approximately -4 cm Hg of pressure, while control cells reached the 1/2 maximum $P_{\text{open}}$ value at approximately -3 cm Hg of pressure. Stretch sensitivity was three times greater in stretched cells than controls, .22 compared to .07 respectively.
Boltzmann equation of experimental findings ($P_{\text{open}}$ vs. pressure)

Figure 7
**Figure 8**: Three types of ion channels encountered in G292 cells. This figure displays traces of the three types of ion channels present in these channels. For all traces, the lowest points represent the baseline or the closed state of the channel. Upward deflections represent ion channel activity to various current amplitudes. First, the small ion channel (approximately 20 pS) is displayed in the first three traces. Second, the intermediate channel (approximately 80 pS) is presented in traces 4-6. Traces 7-9 show the large ion channel (approximately 200 pS), with an underlying intermediate channel. Most traces included more than one type of channel. In fact, the majority of recordings had at least two channels present at some point in the record.
Three types of ion channels encountered in C292 cells

1. the small ion channel (-20 pS)
2. the intermediate ion channel (-80 pS)
3. the large ion channel (-200 pS)
**Figure 9A:** Sample traces from stretched cells with applied pressure.

This series of traces shows one large ion channel with an underlying intermediate channel. Again, the lowest points in the trace represent the baseline or closed state of the channel. Upward deflections identify ion channel activity in an open state. In this series of traces, the first channel which appears to open is the intermediate channel. At -2 cm Hg of pressure, the large ion channel started to open along with the underlying intermediate channel. The intensity of ion channel opening increased progressively at -3 and -4 cm Hg of pressure, with the intensity being the greatest in the last trace. The patch in this cell did not last beyond -4 cm Hg of applied negative pressure. The scale in the lower right corner helps to identify the current amplitude over time.
Sample traces from stretched cells with applied pressure

one large ion channel with an intermediate channel

Figure 9A
**Figure 9B:** Sample traces from stretched cells with applied pressure.

This series of traces identifies two large ion channels with an intermediate channel. Again the lowest points in the trace represent the baseline or closed state with no channel activity. Upward deflections indicate channel activity. Initially under 0 and -1 cm Hg of pressure, a single intermediate ion channel was present. At -2 cm Hg of pressure, two large ion channels open. At times only one channel is open, identified by the upward deflection found in the middle of the trace. The trace at this pressure is not completely clear as many traces tend to be when the channel first opens. A much clearer representation of the two channels is apparent at traces with -3, -4, and -5 cm Hg of applied negative pressure. Again the uppermost deflection of the trace represents both channels in the open state. Generally, as more negative pressure was applied, the channels tended to stay in the open state for a longer period of time. Obviously, the baselines in these last three traces is virtually non existent. The scale of current amplitude over time is present in the lower right corner.
Sample traces from stretched cells with applied pressure

two large ion channels with an intermediate channel

0 cm Hg pressure

-1 cm Hg pressure

-2 cm Hg pressure

-3 cm Hg pressure

-4 cm Hg pressure

-5 cm Hg pressure

Figure 9B
Figure 9C: Sample of a typical control cell. This figure displays three large ion channels with both small and intermediate channels. Again, the baseline is denoted by the lowest points of the trace, while upward deflections identify ion channel activity. At the time of the patch, a small channel was present with a quick, single opening of a large channel. This small channel persisted to some degree up through -3 cm Hg of pressure. At -4 cm Hg of pressure, an intermediate channel and two large channels were apparent, with an obvious loss in small channel readings. The intermediate channel had much more activity than the two large channels, which were just beginning to open. The trace at -5 cm Hg of pressure clearly shows that three large ion channels were present with the majority of the trace being in the open state for two or three of the channels. Almost total absence of the baseline is noted. Some distortion was also present as evidenced by uneven jumps in the trace. This commonly occurs just before the patch is broken. The scale is present in the bottom right corner.
Sample of a typical control cell

three large ion channels with both small and intermediate channels

Figure 9C
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