Bisphosphonates stimulate an endogenous nonselective cation channel in *Xenopus* oocytes: potential mechanism of action

Weijian Shao, Roy C. Orlando, and Mouhamed S. Awayda

Department of Physiology, Tulane University Health Sciences Center, and Veterans Affairs Medical Center, New Orleans, Louisiana

Submitted 10 August 2004; accepted in final form 14 March 2005

Bisphosphonates stimulate an endogenous nonselective cation channel in *Xenopus* oocytes: potential mechanism of action. Am J Physiol Cell Physiol 289: C248–C256, 2005. First published March 23, 2005; doi:10.1152/ajpcell.00393.2004.—The mechanisms of action of bisphosphonates (BPs) have been poorly determined. Besides their actions on osteoclasts, these agents exhibit gastrointestinal complications. They have also recently been described as affecting various preparations that express an epithelial Na\(^+\) channel (ENaC). To understand the effects of BP on ion channels and the ENaC in particular, we used the *Xenopus* oocyte expression system. Alendronate and, similarly risedronate, two aminobisphosphonates, caused a large stimulation of an endogenous nonselective cation conductance (NSCC). This stimulation averaged 63 ± 12 μS (n = 18) 60 min after the addition of 2 mM alendronate. The effects on the endogenous NSCC were blocked by extracellular acidification to pH 6.4. On the other hand, alendronate caused a small inhibition of ENaC conductance at pH 7.4 and 6.4, but the effects at pH 6.4 were more readily observed in the absence of changes of the endogenous conductance. The effects on membrane capacitance were also markedly different, with a clear decrease at pH 6.4 and no consistent changes at pH 7.4. The effects on the endogenous channel were further augmented by genistein and were inhibited by a tyrosine phosphatase inhibitor, indicating the involvement of the tyrosine kinase pathway. Stimulation of NSCC with BP is expected to cause membrane depolarization and may explain, in part, its mechanisms of action in inhibiting osteoclasts.

**BISPHOSPHONATE (BP)** is a common course of therapy for the treatment of osteoporosis. These agents target osteoclasts and inhibit their ability to resorb bone. Their molecular mechanism of action is partially defined. Recently, a variety of effects have been attributed to BP treatment. These actions include long-term cell growth effects that range from activation of caspases and initiation of apoptosis (7) to inhibition of protein prenylation, leading to effects on various GTP-binding proteins such as Ras (18). These effects are observed in a variety of cell models, including Caco-2 cells, a gastrointestinal (GI) cell line model (25). Additional reported effects include changes in cell signaling by stimulation of protein tyrosine phosphatase (PTP) activity (23). These actions can lead to effects on the cell cycle and may explain, in part, the added effects of BPs in inhibiting tumor growth and the potential usefulness of BPs as chemotherapeutics (see Ref. 20).

Besides their ability to inhibit osteoclast growth and/or function, it is now apparent that BPs exhibit additional effects on various other organ systems. Many of these systems are epithelial in nature. For example, Vasikaran et al. (26) found that alendronate affects renal tubule phosphate reabsorption. Similarly, it is well established that GI irritation is a common side effect of BP treatment. An interesting finding regarding GI irritation with BP is that these effects are enhanced in alkaline environments (9). These findings indicate separate effects of BP on membrane transport as well as paracellular transport processes, especially in GI epithelia, in which a barrier function is dependent on an intact (high resistance) paracellular pathway (for review, see Ref. 21). Indeed, these findings also are supported by our data representing the first evidence for the effects of these agents on ion transport in a native epithelium (13).

It is uncertain whether the effects on cell signaling and caspases result from or lead to the modulation of membrane ion channel activities. However, given the role of ion channels and membrane transport in cellular homeostasis, the effects of BP on ion channels may represent the missing link in understanding their mechanisms of action in a variety of systems, including bone cells. For example, osteoclast function is highly dependent on Ca\(^{2+}\) sensing through a Ca\(^{2+}\) receptor. Activation of this receptor leads to an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), leading to cell inhibition. This activation is dependent on membrane depolarization through the activation of a nonselective cation channel (31). This stimulation can also be mimicked by membrane depolarization with high extracellular K\(^+\) (19, 22). Therefore, the effects of BP on membrane voltage and membrane ion channels are of great importance in understanding the mechanism by which they induce their actions in various preparations.

We recently described the effects of alendronate on ion transport in esophageal epithelia (13). Because we now think that the native channel in these cells is a nonselective cation channel that may contain epithelial Na\(^+\) channel (ENaC) subunits as part of its core (5), we have investigated the effects of BPs in the *Xenopus* oocytes expression system. This system is ideal for these experiments because it contains endogenous nonselective cation channels (2, 30) and also allows the exogenous expression of the cloned ENaC. Moreover, by virtue of its simplicity, this system allowed us to discern electrophysiological effects on membrane ion channels, a measurement that is complicated by the contribution of the paracellular pathway in epithelial preparations. This system also allowed us to monitor membrane capacitance continuously and to assess the potential effects of these agents on the membrane area and/or the dielectric coefficient (3, 6).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
We report that alendronate and, similarly, risedronate induced marked stimulation of an endogenous nonselective cationic conductance (NSCC) in Xenopus oocytes. This stimulation was essentially abolished at pH 6.4. These effects were not accompanied by appreciable changes of membrane capacitance, indicating the absence of a major effect on membrane trafficking. In contrast, these agents caused an inhibition of ENaC currents and conductance. The effects on the endogenous NSCC were likely mediated via effects on the tyrosine kinase pathway, because they were further stimulated in the presence of a tyrosine kinase inhibitor and highly attenuated by blocking tyrosine phosphatases. These data provide the first mechanistic evidence for an effect of BPs on a membrane ion channel. These effects differed between ENaC and the endogenous NSCC, providing an underlying mechanism for the diversity in the actions of BPs in various preparations. Membrane depolarization observed with stimulation of a NSCC may provide a potential mechanism for the actions of these agents in bone cells.

MATERIALS AND METHODS

Xenopus oocytes. Oocytes were processed as previously described by Awayda (3). Briefly, oocytes were surgically removed and defolliculated with collagenase (type 1A; Sigma Chemical, St. Louis, MO) in a nominally Ca2+-free solution. Defolliculated oocytes were allowed to recover overnight before injection or use in electrophysiological studies. Under these conditions, the background conductance in control untreated oocytes was low and <1 μS. In some experiments, oocytes were injected with cRNA for rat α-, β-, and γ-ENaC. These oocytes were recorded from 1 to 5 days after injection. These oocytes exhibited a higher conductance that was blocked (>98%) by a low dose of amiloride (20 μM).

Solutions used for oocyte incubation and recording were as previously described (3). Solution composition was (in mM) 94 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4. For the low-Na+ solution, 89 mM NaCl was replaced with NMDG-Cl− or with KCl. Alendronate was obtained from Merck Pharmaceuticals (Rahway, NJ) and was dissolved directly into the recording solution. pH was adjusted using NaOH. The Na+ concentration of the alendronate solution was determined using a flame photometer. Genistein was used at 100 μM as a broad-spectrum tyrosine kinase inhibitor. PTP inhibitor IV (PTPI-IV) or bis(4-trifluoromethylsulfonylaminomethyl)-1,4-disopropylbenzene was used at 20 μM. All of these reagents were obtained from Calbiochem (EMD Biosciences, San Diego, CA).

Impedance analysis. In all experiments, a Dagan TEV 200 oocyte voltage clamp was used (Dagan, Minneapolis, MN). Impedance was continuously measured every 10 s as described by Awayda (3). The measurement consisted of five sequential, discrete sine wave signals ranging from 55 to 390 Hz. These signals were in the range of 2 mV from peak to peak. Impedance analysis allows the assessment of membrane capacitance (Cm), membrane conductance (Gm), and membrane current (Im). Capacitance is classically accepted to reflect membrane area, and therefore changes of this parameter can reflect effects on area or membrane dielectric coefficient (3, 6). Changes in Cm indicate membrane interaction or vesicular traffic-mediated events. Membrane conductance was measured at 0 mV, which is near the reversal potential of ENaC-expressing oocytes and those with stimulated NSCC activity. In this regard, this maneuver allowed us to perform long experiments without major effects on the oocyte intracellular ionic composition. Moreover, because the impedance measurements were performed with small-amplitude sinuosities, the resulting conductance represented the slope conductance at the holding voltage. This conductance is unaffected by changes in reversal potential, in contrast to that of membrane current, and represents an ideal way to examine effects on ion channel activity. In most cases, Im was low and close to 0, given that the holding potential was close to that of the reversal potential. However, measurements of Im were a useful indicator of the direction of current flow and in assessing the initial changes that occurred in oocyte membranes, especially in response to ionic composition changes (see RESULTS).

Current-voltage (I-V) relationships were obtained by clamping the membrane potential to voltages between −100 and +40 mV (cytoplasmic with respect to ground). All voltages were returned to 0 mV to ensure the stability of these measurements and to rule out effects on intracellular ionic composition. Statistical significance was determined using Student’s t-test, with P < 0.05 considered a significant difference.

RESULTS

Alendronate, and to a similar extent risedronate, caused a shift of the pH of ND94. At a concentration of 4 mM, these BPs decreased pH to 6.4. In initial experiments, we titrated the pH back to control values (pH 7.4). These titrations were performed using NaOH and did not appreciably alter the solution osmolality.

Effects on endogenous currents. As shown in Fig. 1, the baseline slope conductance (Gm) in control oocytes was very low and in the range of 1 μS. Addition of 2 mM alendronate at pH 7.4 caused a gradual increase of conductance. An initial plateau was reached within ~15 min. Invariably, the effects were biphasic in nature, and a secondary increase was observed at time points up to 60 min. We focus herein on the effects on conductance, because this parameter is independent of changes of reversal potential (see MATERIALS AND METHODS). Moreover, the changes in current (Im) were small, owing to the holding potential of 0 mV, which is close to the reversal potential of the alendronate-stimulated channel (see Fig. 6 and Table 1).

Addition of a low-Na+ solution in which ~85 meq Na+ was substituted with NMDG+ caused a large (~80%) decrease in Gm, indicating that the alendronate-stimulated conductance was indeed cationic in nature. In all experiments, the effects of alendronate were reversible and both conductance and current
returned to control values within 10–20 min after washout. These effects occurred in the absence of changes to \( G_m \), indicating no effects on membrane area or dielectric properties.

Because of our interest in examining the effects of alendronate on ENaC, and because of the apparent cationic selectivity of this channel, we examined the effects of amiloride. As shown in Fig. 2, 20 \( \mu \)M amiloride, a concentration that completely blocks ENaC, caused an appreciable inhibition of this cationic channel. This conductance was further inhibited by substituting the large cation NMDG\(^+\) for Na\(^+\). Similar to the results shown in Fig. 1, these effects on \( G_m \) were observed in the absence of appreciable changes in \( C_m \).

It is unlikely that this endogenous channel is ENaC, owing to the absence of an appreciable amiloride effect in control untreated oocytes. Moreover, 20 \( \mu \)M amiloride only partially blocked this channel, while this concentration is known to produce a complete block of ENaC. In addition, there were no differences in the response to alendronate in cells pretreated with amiloride to block any endogenous ENaC (Fig. 2, bottom). Given these findings, this channel is likely related to the endogenous maitotoxin- and DIDS-stimulated cationic channel described by Diakov et al. (12) because it is known to be blocked by millimolar concentrations of amiloride. The link to this NSCC is further described below.

The effects of alendronate on control oocytes are summarized in Table 1. Similar but appreciably smaller effects were also observed with 200 \( \mu \)M alendronate. Therefore, we focused on the large effects caused at 2 mM and summarized the data at 15 and 60 min. At 60 min, \( G_m \) increased to 64.9 \( \mu \)S, an \( \sim \)46-fold stimulation over the control untreated values. A threefold increase of \( I_m \) was observed. This minimal increase provides a further indication that the holding voltage of 0 mV is close to the reversal potential (\( E_{rev} \)) of the stimulated current (at a holding voltage of exactly \( E_{rev} \), there is no current). This further strengthens the idea that this stimulated conductance is a nonelective cation channel.

The changes summarized above occurred in the absence of significant effects on \( C_m \). The interpretation of this finding is twofold. First, this stimulation is unlikely to be due to interaction between BPs and the membrane dielectric, because this likely would have resulted in changes in \( C_m \) (6). Second, this stimulation rules out membrane trafficking events and indicates actions of alendronate on membrane-resident channels. These data are consistent with the magnitude of endogenous channel.

### Table 1. Effects of alendronate on ion channels in Xenopus oocytes at pH 7.4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( C_m ), nF</th>
<th>( G_m ), ( \mu )S</th>
<th>( I_m ), ( \mu )A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, ( n = 18 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>237±8</td>
<td>1.4±0.1</td>
<td>0.154±0.028</td>
</tr>
<tr>
<td>15 min</td>
<td>229±15</td>
<td>22.0±3.7*</td>
<td>2.37±0.041*</td>
</tr>
<tr>
<td>60 min</td>
<td>238±8</td>
<td>64.9±12.3*</td>
<td>2.46±0.114*</td>
</tr>
<tr>
<td>ENaC, ( n = 16 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>250±11</td>
<td>33.1±7.2</td>
<td>-0.119±0.033</td>
</tr>
<tr>
<td>15 min</td>
<td>300±49</td>
<td>76.2±11.0*</td>
<td>0.170±0.054*</td>
</tr>
<tr>
<td>60 min</td>
<td>243±15</td>
<td>66.7±10.6*</td>
<td>0.187±0.047*</td>
</tr>
</tbody>
</table>

Data are means ± SE; \( n \), no. of experiments. \( C_m \), membrane capacitance; \( G_m \), membrane conductance; \( I_m \), membrane current; ENaC, epithelial Na\(^+\) channel. *\( P < 0.05 \), statistically different from paired values at 0 min.

Fig. 2. Amiloride and low Na\(^+\) inhibit the alendronate-stimulated endogenous channel. See Fig. 1 legend for additional details. Addition of 20 \( \mu \)M amiloride caused an appreciable but incomplete inhibition of the alendronate-stimulated current and conductance. This was further inhibited by reducing [Na\(^+\)]\(_o\) to 5 mM. Changes in \( G_m \) occurred in the absence of changes of \( C_m \) (bottom). Pretreatment with amiloride did not affect the response to alendronate, indicating that both components of the biphasic stimulation are sensitive to amiloride. Solution pH was adjusted to 7.4. Data represent 8 and 6 experiments in the top two images and bottom image, respectively. Table 1 summarizes data shown in Figs. 1 and 2.

Fig. 3. Effects of alendronate on conductance in epithelial Na\(^+\) channel (ENaC)-expressing oocytes. Control or ND94 solution is referred to as C. Amiloride (20 \( \mu \)M) was added at the beginning and end of each experiment (A or Amilo). The amiloride-inhibitable conductance observed at the beginning represents that of ENaC, while that at the end of the experiment represents the cumulative effect on ENaC and the endogenous channel. Top trace shows that \( G_m \) exhibited little or no secondary increase after 15 min of alendronate treatment. Bottom trace shows that \( G_m \) exhibited a secondary decrease after the first 15 min of alendronate. Solution pH was adjusted to 7.4. Data are representative of 16 experiments. See Table 1 for data summary.
expression of ENaC (see Fig. 3A). Similar effects on ENaC using other BPs, we tested the effects of risedronate (data not shown) in a system that may contain a potentially large number of evidence for marked differences in the response of ENaC and the endogenous cationic channel to BP. To better understand the Importance of considering both negative and positive controls to the response shown in Figs. 1 and 2, addition of alendronate at pH 6.4 was without sustained effects on conductance. A small decrease in $C_m$ was observed. This was also observed with pH 6.4 alone (see Table 2 and text). Data are representative of 9 experiments.

Effects of alendronate on ENaC expressed in Xenopus oocytes

To determine whether similar effects could be observed using other BPs, we tested the effects of risedronate (data not shown). Similar effects on $G_m$, $C_m$, and $I_{NSCC}$ were observed. These changes were also largely inhibited by reducing extracellular $[\text{Na}^+]$, indicating a cationic selectivity of the stimulated conductance. These changes were reversible after washout of risedronate. These data indicate a class effect of amniobisphosphonates on an endogenous cationic channel. Given these findings, we concentrated our efforts on alendronate.

Effects on ENaC-expressing oocytes. As with control oocytes, alendronate caused a large change in $G_m$ in ENaC-expressing oocytes (see Fig. 3A). A similar increase was observed that exhibited an initial plateau within $\sim 15$ min. However, the time courses of the secondary changes were consistently different between these two groups of oocytes. The secondary stimulation observed in control oocytes at intervals between 15 and 60 min was absent. Instead, either no appreciable additional change (Fig. 3A) or a secondary decrease in $G_m$ (Fig. 3B) was observed. These effects are summarized in Table 1, showing that, on average, a small decrease in $G_m$ between 15 and 60 min was observed.

One potential explanation for this finding is the presence of a delayed inhibition of ENaC superimposed on the secondary stimulation of an endogenous cationic channel. Because the endogenous channel is partially amiloride sensitive, the changes with amiloride in Fig. 3 reflect the sum of the effects on the endogenous channel as well as on ENaC. Qualitatively, the effects of amiloride at 60 min in ENaC-expressing oocytes were smaller than those expected on the basis of the sum of the ENaC amiloride-sensitive component observed before adding alendronate and the amiloride-inhibitable component of the endogenous channel, thus providing support for the above interpretation (see also below). However, despite difficulties in assessing each of these components, it is clear that alendronate did not stimulate ENaC. This indicates specific effects on the endogenous cationic channel. Given that acidic pH is reported to ameliorate the GI complications observed with BPs (9), we tested the effects of these agents at pH 6.4.

Effects at acidic pH. Addition of alendronate at pH 6.4 caused a small, transient stimulation of $G_m$ (Fig. 4). Within 10 min, $G_m$ returned to values not different from control. In contrast, a small decrease in $C_m$ was observed in these experiments that was similar to that observed with pH 6.4 alone (Table 2). Thus the effects on the endogenous cation channel are completely dependent on extracellular pH. This finding may explain, in part, the reduction in GI complications at acidic pH. Keeping in mind that the ENaC expressed in oocytes is minimally affected by a small decrease in extracellular pH (Refs. 4, 10; see also Table 2), we took advantage of the lack of effects on the endogenous NSCC at pH 6.4 to examine the separate effects on ENaC.

As shown in Fig. 5, alendronate at pH 6.4 caused a large decrease in $G_m$ in ENaC-expressing oocytes. This effect could not be explained by pH 6.4 alone and was completely reversible upon washout of alendronate. Alendronate at pH 6.4 also caused a decrease in $C_m$ similar to that observed with pH 6.4 alone. These changes are summarized in Table 2. The changes in $G_m$ with alendronate were larger than the changes in $C_m$ (53% vs. 23%), indicating specific additional effects of this compound on membrane-resident ENaC (3).

It is uncertain whether the pH-dependent effects of alendronate on the endogenous channel are due to the sensitivity of the channel to pH or to effects on the ionization status of alendronate itself. BPs exhibit five ionization states with dissociation constants ($pK_a$) for the four transition states of 1.35, 2.87, 7.03, and 11.3. Thus a difference in ionization was expected between pH 7.4 and 6.4 for the group with a $pK_a$ of 7.03. Despite the fact that the effects on ENaC do not appear to have been affected by this shift in ionization, we cannot rule out this possibility as an explanation for the lack of sustained endogenous channel stimulation. In either case, the data provide clear evidence for marked differences in the response of ENaC and the endogenous cation channel to BP. To better understand the

Table 2. Effects of alendronate on ENaC expressed in Xenopus oocytes

<table>
<thead>
<tr>
<th>pH 6.4</th>
<th>pH 6.4, Alendronate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>$C_m$, $\mu F$ (n = 9)</td>
<td>299±7</td>
</tr>
<tr>
<td>$G_m$, $\mu S$ (n = 9)</td>
<td>31.7±6.0</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 9 experiments. E/C, experimental divided by control. *P < 0.05, statistically significant difference from paired control data. †P < 0.001, statistically significant difference from E/C values in pH 6.4 group.
Fig. 5. Inhibition of ENaC by alendronate at pH 6.4. Example of the effects of alendronate on an ENaC-expressing oocyte at pH 6.4, at which the stimulation of the endogenous cation channel is eliminated. Changes in both $C_m$ and $G_m$ were reversible upon washout of alendronate. Data are representative of nine experiments. See Table 2 for data summary.

effects of BPs, we focused our attention on the properties and mechanisms of endogenous channel activation.

Activation of a nonselective endogenous channel. The evidence presented above is consistent with alendronate activation of NSCC. Moreover, experiments with low [Na$^+$] demonstrated that the endogenous channel not only exhibits an appreciable Na$^+$ permeability but also is poorly permeable to the large monovalent cation NMDG$^+$. These findings rule out any major contribution of Cl$^-$, because substitution of NMDG$^+$ for Na$^+$ inhibited this conductance by >80%, even in the presence of 5 mM and 2 mM external Na$^+$ and K$^+$. To better describe this conductance, we examined its $I$-$V$ relationship (Fig. 6). A clear feature of the whole cell currents was that they exhibited a time dependence at hyperpolarizing voltages.

This inhibition is easily distinguished from that of ENaC, which exhibits a small activation at those voltages (see Fig. 7 and Ref. 3). As shown in Fig. 6, the kinetics of the whole cell currents were not different between 10 and 60 min after alendronate and indicate activation of the same channel. They also were similar to the kinetics of the channel activated in ENaC-expressing oocytes (Fig. 7), also indicating activation of this channel.

Analysis of the $I$-$V$ relationship in control oocytes (Fig. 6) revealed that alendronate caused a large rightward shift of the membrane reversal potential at both early and late time points. This shift was consistent with activation of a cation channel, specifically one with appreciable Na$^+$ permeability, because $V_m$ is depolarized toward the Na$^+$ equilibrium potential. On average, alendronate caused depolarization of $V_m$ from −58.8 to −6.2 mV ($n = 6$). This value is clearly different from the approximately −40-mV reversal potential for Cl$^-$ and further rules out stimulation of anionic conductance. However, this is also different from the −40- to 50-mV reversal potential for Na$^+$ and indicates permeability to other cations, namely, in this case, K$^+$. This conclusion is consistent with the shift observed in NMDG and the reversal voltage for the NMDG-inhibitable currents. These shifts are consistent with those observed by others who have examined oocyte endogenous NSCC (8, 12, 30). It is also important to point out that the endogenous NSCC described by others exhibits a linear or slightly inwardly rectifying $I$-$V$ relationship similar to that shown in Fig. 6 herein. This is very distinct from that of the endogenous Cl$^-$ channels that exhibit marked outward rectification (Refs. 1, 24, 28; for review, see also Ref. 29).

The endogenous NSCC is known to exhibit a slightly higher K$^+$-to-Na$^+$ permeability (8). To determine whether this is the same as the alendronate-stimulated channel, experiments were performed with K$^+$-for-Na$^+$ substitution. As shown in Fig. 8, addition of the high-K$^+$ solution caused a near-doubling of $G_m$. This was accompanied by a large increase in $I_m$ in the inward direction, consistent with K$^+$ entry. These data indicate a higher permeability for K$^+$ than for Na$^+$. Indeed, this permeability ratio can be estimated from the ratio of the conductances in Na$^+$ and K$^+$ solutions and is found to average 1.93 ± 0.26
(n = 7). This is similar to the K⁺-to-Na⁺ selectivity of the endogenous NSCC reported by others (8).

An interesting feature of the endogenous NSCC studied by others is that it is activated by the disulfonic stilbene DIDS. Therefore, we tested whether DIDS also stimulates the alendronate-activated NSCC. As shown in Fig. 9, addition of 100 μM DIDS was accompanied by a further stimulation of \( G_m \). On average, DIDS increased \( G_m \) from 35.2 ± 9.5 to 54.3 ± 16.1 (n = 5), an ∼54% stimulation. Thus these findings clearly indicate that alendronate stimulates an endogenous NSCC that is electrophysiologically and functionally the same as that described by others (8, 12, 30). This finding may also explain some of the GI complications associated with alendronate because these epithelia are also now known to contain an apical NSCC (5).

**Potential mechanism.** BPs have been reported to enhance tyrosine phosphatase activity (23). To test whether the observed effects of alendronate on the NSCC are mediated via this pathway, we examined the effects of inhibitors of tyrosine kinase and phosphatase pathways. To inhibit tyrosine kinase, we used the broad-spectrum inhibitor genistein. This inhibitor was added at 100 μM 30 min after the addition of alendronate. This time point was chosen because it reflects a period within which \( G_m \) is still capable of a large increase. As shown in Fig. 10, genistein augmented the effects of alendronate by causing a further increase in \( G_m \). This occurred in the absence of appreciable changes in \( C_m \) and was not observed with genistein alone in the absence of alendronate (data not shown).

To further demonstrate the involvement of the tyrosine kinase pathway, we used an inhibitor of PTPs. As shown in Fig. 10, addition of 20 μM PTPI-IV, a broad-spectrum competitive inhibitor of tyrosine phosphatases, nearly abolished the stimulation observed with alendronate. These data provide clear evidence for the participation of this pathway in the effects of BP on the NSCC. Moreover, these data indicate that the entire alendronate-induced stimulation, albeit biphasic, is sensitive to tyrosine phosphatase inhibition.

The effects of genistein and PTPI-IV are summarized in Fig. 11, in which it is clear that genistein more than doubled the \( G_m \).
in alendronate-treated cells, while PTPI-IV decreased $G_m$ by ~88%. Thus it is clear that this NSCC is regulated by tyrosine kinase and that the effects of alendronate are mediated via this pathway. It is unknown whether the reported effects of maitotoxin on the endogenous NSCC in oocytes are genistein sensitive. However, it is interesting to note that the effects of maitotoxin in a variety of other systems are sensitive to genistein (22, 27). Given the similarities between NSCC and the effects of these various agents, it is highly likely that these actions are sensitive to tyrosine kinase and phosphatase.

**DISCUSSION**

We have examined the effects of aminobisphosphonates on endogenous ion channels and ENaC heterologously expressed in *Xenopus* oocytes. We find that BPs stimulate an endogenous NSCC. This observation is in contrast to that observed with the highly Na$^+$-selective ENaC, which was inhibited by these agents. These effects were observed in the absence of BP-induced changes of capacitance, and they were readily reversible. The effects on the endogenous NSCC were abolished at pH 6.4 and were mediated via the tyrosine kinase-phosphatase pathway.

**Effects on the endogenous NSCC.** We provide compelling evidence for stimulation of an endogenous nonselective cation channel by BPs. This channel exhibits a reversal of nearly 0 mV (~6.2 mV), which is in the range of that expected for a channel with appreciable Na$^+$ and K$^+$ permeability. Moreover, substitution of Na$^+$ with NMDG$^+$ caused a marked inhibition of this channel. We think that the NSCC stimulated by BP is the same as that characterized by others in response to stimulation by the marine toxin maitotoxin (8, 12, 30). Both channels are endogenous to oocytes, stimulated by DIDS, and inhibited by high concentrations of amiloride and exhibit permeability sequences of K$^+ >$ Na$^+ >$ NMDG$^+$. While we and others have not presented a complete amiloride dose-response curve for this NSCC, the results are similar. We observed a 20–30% inhibition at 20 μM amiloride, while others have observed a 10–15% block at 10 μM and a 60–70% block at 1 mM (8). However, there is a small difference between our data and those of Bielfeld-Ackermann et al. (8) in terms of K$^+$/Na$^+$ permeability (2:1 vs. 1.5:1, respectively). We think that the main difference for this small discrepancy is that Bielfeld-Ackermann et al. used ratios of current to calculate the permeability, while we used ratios of conductance. Because currents are sensitive to reversal potential changes, we think that conductance represents a more appropriate measurement.

An interesting finding was that these effects were highly dependent on pH. We are uncertain whether the pH dependence of the effects of BP on the NSCC is due to effects of pH on this channel or on BP. We favor the idea that these are effects of pH on the NSCC, because the effects on ENaC are still observable at this pH. A second possibility is that pH inhibits the second-messenger kinase-phosphatase affected by BP. However, given that this pH did not affect the inhibition of ENaC, one would also have to invoke different mechanisms of action on the two channels. At present, we cannot distinguish these possibilities.

**Effects on ENaC.** Amiloride at a concentration of 10–20 μM causes a nearly complete block of ENaC and reduces the conductance and whole cell currents of ENaC-expressing oocytes to values very similar to those observed in control untreated oocytes. Thus ENaC activity in untreated oocytes can be assessed from the magnitude of the amiloride-inhibitable conductance. However, the amiloride-sensitive conductance in ENaC-expressing oocytes was smaller after treatment with BP than that observed at the beginning of the experiment. Combined with the fact that the value after BP addition is overestimated owing to the small block of the NSCC, these findings
indicate inhibition of ENaC. Taking advantage of the observations that the effects of BP on the endogenous NSCC were pH dependent and that the effects of pH 6.4 alone on ENaC were minimal, we were able to examine the effects on ENaC using amiloride uncomplicated by the contribution of the endogenous channel. In these experiments, we demonstrated that BP caused marked inhibition of ENaC (see Table 2).

Mechanism of action of BP-tyrosine kinase-phosphatase. The mechanisms of actions of BPs are poorly defined but recently have been elucidated to involve second-messenger signaling cascades that lead to changes in protein prenylation and tyrosine phosphatase activity, among others (18, 23). It is reasonable to assume that the changes in cellular function are mediated by changes in the intracellular milieu. Moreover, it is also highly likely that the changes in the intracellular milieu are mediated by or lead to changes in the activity of membrane-bound ion channels and transporters. With the exception of a previous report documenting effects of BP on vacuolar H+/ATPase in isolated chicken osteoclast vesicles (11) and our previous findings in esophageal epithelia (13), such hypotheses have not been confirmed. Therefore, the present report describes the first detailed findings of effects on an ion channel.

Our findings are consistent with a second-messenger tyrosine phosphatase-mediated stimulation of the NSCC. These findings are also consistent with the effects observed in esophageal epithelia, in which the native channels in the apical membrane are also NSCC (5) and, moreover, were stimulated by BP (13). These data represent the first observation and second-messenger explanation of the effects of BP on membrane ion channels. The similarities of the effects in diverse systems such as frog oocytes and native rabbit esophagus argue in favor of a generalized phenomenon that is likely to be observed regarding NSCC in a variety of cell membranes. It is unclear whether the effects on these channels precede or are mediated via changes of cell signaling; however, our findings with the tyrosine kinase and tyrosine phosphatase inhibitors argue in favor of the latter hypothesis.

Relevance to the in vivo effects of BP. The tyrosine kinase signaling pathway is a ubiquitous system observed in many cell types, including osteoclasts. Many cells, including osteoclasts and osteoblasts, also express NSCC. These channels may play an important role in regulating membrane voltage and may also be responsive to mechanical forces (16). When activated, these channels lead to membrane depolarization dominated by the large difference between resting membrane potential and the Na+ equilibrium potential. This depolarization is likely to activate the Ca2+ receptor, leading to increased [Ca2+]i, and osteoclast inhibition (19, 22, 31). It is interesting to note that the increase in Ca2+ and potentially in membrane depolarization in these cells is mimicked by inhibition of protein tyrosine kinase (15). Activation of a Na+ and K+ conductance could also favor apoptosis through membrane depolarization or the decrease of intracellular K+ as observed in a variety of preparations (14, 17). Similarly, apoptosis could be favored by the ensuing cell shrinkage, a classic activator of this process. In either case, stimulation of a Na+- and K+-conductive pathway may explain the diverse effects of BP in different preparations, especially bone cells.

The stimulation of the NSCC with alendronate was observed at concentrations as low as 200 μM (data not shown). However, this stimulation was much smaller than the effect observed at 2 mM. While this concentration appears high, it is possible that this is due to a higher threshold of effects of BP on the oocyte endogenous tyrosine kinase. Indeed, it is well known from another kinase system, the PKA system, that *Xenopus* oocytes exhibit different sensitivities to stimulation, owing to a highly active endogenous phosphodiesterase system. Moreover, it is also important to keep in mind that BPs exhibit a very high affinity and are able to bind to hydroxyapatite. Thus the actual concentration encountered by osteoclasts may be much higher than that observed in plasma.

**REFERENCES**


