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Antiallodynic effect of etidronate, a bisphosphonate, in rats with adjuvant-induced arthritis: Involvement of ATP-sensitive K^+ channels

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Abstract

Bisphosphonates, pyrophosphate analogues, known as inhibitors of bone resorption, appear to cause analgesia in certain clinical painful situations. To detect clinically relevant analgesic property of etidronate, a non-aminobisphosphonate, we examined and characterized its antiallodynic effect in the rat with adjuvant-induced arthritis, in comparison with alendronate, an aminobisphosphonate, as determined by the von Frey test. Repeated systemic administration of etidronate at 10–40 mg/kg/day suppressed the adjuvant-induced mechanical allodynia in rat hindpaw, an effect reaching a plateau in approximately 10 days. Systemic or intraplantar (i.pl.) administration of ATP-sensitive K^+ (K^+_{ATP}) channel inhibitors, glibenclamide and/or tolbutamide, completely reversed the antiallodynic effect of etidronate within 1 h in the arthritic rats, without affecting the nociceptive scores in naïve or arthritic animals that had not received etidronate. Alendronate, administered repeatedly, also revealed similar glibenclamide-reversible antiallodynic effect. In contrast, the antiallodynic effect of repeated systemic indomethacin was resistant to i.pl. glibenclamide in the arthritic rats. Repeated administration of etidronate or alendronate only slightly attenuated the adjuvant-evoked hindpaw edema. Among K^+_{ATP} channel subunits, mRNAs for Kir6.1, SUR1, SUR2A and SUR2B were abundant in rat dorsal root ganglia, while Kir6.2 mRNA was poor. Our data demonstrate that repeated etidronate as well as alendronate exhibits antiallodynic activity in arthritic rats, which might be clinically relevant, and suggest involvement of K^+_{ATP} channels in the underlying mechanisms.

Keywords: Etidronate; Bisphosphonate; Allodynia; Pain; Analgesia; Adjuvant

1. Introduction

Bisphosphonates are a class of pyrophosphate analogues that contain a phosphate-carbon-phosphate (P-C-P) backbone, being applicable to the treatment of a number of common bone diseases such as Paget's disease, tumor-associated osteolysis and postmenopausal osteoporosis. The first generation of bisphosphonates, i.e., non-aminobisphosphonates, including etidronate and clodronate might inhibit bone resorption by affecting functions of osteoclasts through the intracellular accumulation of a cytotoxic, β-γ-methylene (AppCp-type) analog of ATP as a metabolite, while the second-generation bisphosphonates, i.e., aminobisphosphonates, including alendronate would act by inhibiting protein isoprenylation (Benford et al., 1999).

There is evidence that bisphosphonates such as etidronate and clodronate are capable of reducing inflammation in vivo most likely by affecting functions of macrophages (Barrera et al., 2000; Harada et al., 2004; Oelzner et al., 2000). Further, clinical evidence implies that several bisphosphonates including etidronate, alendronate, clodronate and pamidronate relieve pain in patients with osteoporosis (Storm et al., 1990), osteoarthritis (Forys, 1991) or bone metastasis due to prostate carcinoma (Adami, 1997), and also reduce spontaneous pain complicating reflex sympathetic dystrophy syndrome (RSDS) (Adami et al., 1997; Kubalek et al., 2001). The pain relief caused by the

Abbreviations: BMD, bone mineral density; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; K^+_{ATP}, ATP-sensitive K^+; P-C-P, phosphate-carbon-phosphate; RSDS, reflex sympathetic dystrophy syndrome; RT-PCR, reverse transcription-polymerase chain reaction.

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bisphosphonate in RSDS patients does not appear to be related with the inhibitory effects on osteoclasts (Varenna et al., 2000). Thus, both non-amino- and amino-bisphosphonates are considered analgesic in clinical painful situations particularly related to bone pathology. Of note is that, in some instances, these agents may reduce systemic signs of inflammation in patients with joint inflammation (Goldring and Gravallese, 2004). In animal experiments, acute analgesic effects of several bisphosphonates, when administered i.v. and/or intracerebroventricularly (i.c.v.), have been detected, as assessed by the tail flick test and/or phenylquinone-induced writhing test (Bonabello et al., 2001, 2003). In these studies, however, etidronate and alendronate exhibited only weak antinociceptive activity, while pamidronate and clodronate produced relatively potent antinociception. An independent study (Goicoechea et al., 1999) has shown that alendronate suppressed the acetic acid-induced writhing responses, but had no effect on the formalin-evoked nociceptive responses in the hindpaw. Thus, some of bisphosphonates appear capable of suppressing nociception not related to bone pathology, by central and/or peripheral mechanisms in laboratory animals, which is not necessarily consistent with clinical characteristics of their analgesic effects. Particularly, it is to note that clinical analgesic effects of bisphosphonates can be obtained only after their repeated administration for several days or a few weeks, but not by single administration. Further, the cellular or molecular mechanisms responsible for the analgesic effects of bisphosphonates have yet to be investigated in depth.

In the present study, to detect clinically relevant analgesic property of etidronate, a non-aminobisphosphonate, we used an adjuvant-induced allodynia model in the rat. To explain the mechanisms for the antiallodynic effect of etidronate that could be metabolized to an AppCp-type analog of ATP, we further investigated involvement of ATP-sensitive K^+ (K^+_{ATP}) channels, known to participate in both central (Lohmann and Welch, 1999) and peripheral (Sachs et al., 2004) analgesic mechanisms. We also evaluated and characterized the antiallodynic activity of alendronate, an aminobisphosphonate, for comparison. Here we report that not only etidronate but also alendronate, when administered repeatedly, inhibit adjuvant-induced allodynia in rats through activation of K^+_{ATP} channels in the periphery.

2. Methods

2.1. Animals

Male Lewis rats at 6 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed on a 12:12-h light/dark cycle in a temperature- and humidity-controlled room, and allowed free access to food and water. All experimental procedures were approved by the Kinki University School of Pharmaceutical Sciences' Committee for the Care and Use of Laboratory Animals, and the studies described here adhered to the guidelines of the Committee for Research and Ethical Issues of IASP.

2.2. Assessment of mechanical nociception and edema in rat hindpaw

The sensitivity of rat hindpaw to mechanical stimulation was assessed by use of von Frey filaments with distinct strengths, 10, 26 and 60 g (Touch-Test Sensory Evaluator Instructions, North Coast Medical, Morgan Hill, CA). A rat was placed on a raised wire mesh floor (25 cm high), under a clear plastic box (38 × 33 × 18 cm), and acclimated to the experimental environment for 15 min. The planta pedis of the left hindpaw of each rat was stimulated with the above-mentioned three distinct filaments in an ascending order of the strength. Stimulation with each filament was applied 5 times at 5–10 s intervals, and the behavioral responses were observed and scored. Scoring of nociceptive behavior was defined as follows: score 0 = no response; score 1 = immediate lifting of the stimulated paw or walking; score 2 = licking, biting or flinching of the hindpaw. The data are expressed as the average score of responses caused by 5-time challenges with each filament. In our preliminary experiments, a von Frey hair with strength of 10 g caused little nociceptive behavior in intact rats, while the other two von Frey hairs with strength of 26 or 60 g produced nociceptive responses in the same animals (Fig. 1A). To detect adjuvant arthritis-induced allodynia, therefore, we decided to use the von Frey hair with 10 g strength throughout the present experiments. Paw thickness was measured as an indicator of edema after determination of mechanical nociception.

2.3. Induction of adjuvant arthritis accompanied by allodynia in rat hindpaw

Adjuvant arthritis was induced by single intraplantar (i.pl.) administration of 0.2 ml adjuvant solution containing 1 mg heat-killed *Mycobacterium butyricum* (suspended in liquid paraffin), in the right hindpaw of each rat, as described previously (Harada et al., 2004). Control animals received i.pl. injection of the vehicle (liquid paraffin). Mechanical nociception in the contralateral (left) hindpaw was determined every 2–4 days. The extent of inflammation was monitored by measuring paw thickness with a micrometer (resolution 0.01 mm). Care was taken to assure that the micrometer was placed at the same site on the paw for each measurement and at a similar location across all animals. The nociceptive score in response to 10-g von Frey hair gradually elevated and reached a plateau 3 weeks after adjuvant injection, the evoked allodynia lasting for 3 weeks (until 6 weeks after adjuvant) or more (Fig. 1B). Similarly, edema in bilateral hindpaws developed in parallel with allodynia (data not shown). Therefore, the effect of bisphosphonates on the adjuvant-evoked allodynia was determined 3–6 weeks after challenge with adjuvant.

2.4. Administration of bisphosphonates and indomethacin to the rat with adjuvant-induced arthritis

To detect clinically relevant analgesic effects of bisphosphonates, we examined their effect on the established allodynia in rats. Etidronate, a non-aminobisphosphonate, at 10 or 40 mg/kg (dissolved in saline) was administered s.c. repeatedly, once daily for 10 days, to the rat, approximately 3 weeks after adjuvant challenge. Alendronate, an aminobisphosphonate, at 1 mg/kg (dissolved in saline) was administered s.c. in the same manner. Indomethacin at 5 mg/kg (dissolved in 4% sodium bicarbonate), as described elsewhere (Kato et al., 2002; Saito et al., 2003), was administered s.c. only once or repeatedly once daily for 10 days. Control rats received s.c. administration of the same volume of each vehicle.

2.5. Inhibition experiments

The rat received s.c. and i.pl. administration of glibenclamide, an K^+_{ATP} channel inhibitor, at 10 mg/kg in a volume of 200 μl/kg and 160 μg/paw in a volume of 10 μl (dissolved in DMSO), respectively, on the day after daily treatment with the bisphosphonates or indomethacin for 10 days. Tolbutamide at 160 μg/paw was also administered i.pl. to the rat in the same manner. The appropriateness of the doses and administration schedules employed, in terms of selectivity, potency and toxicity, has been reported previously (Pacheco and Duarte, 2005; Proks et al., 2002; Sachs et al., 2004; Soares and Duarte, 2001; Takasaki et al., 2004), and no toxicity was apparently observed in the preliminary experiments.

2.6. Analysis of mRNAs for K^+_{ATP} channel subunits in rat dorsal root ganglia by reverse transcription-polymerase chain reaction

To analyze K^+_{ATP} channel subunits expressed in the primary afferent neurons of rats, we examined mRNAs for Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B in

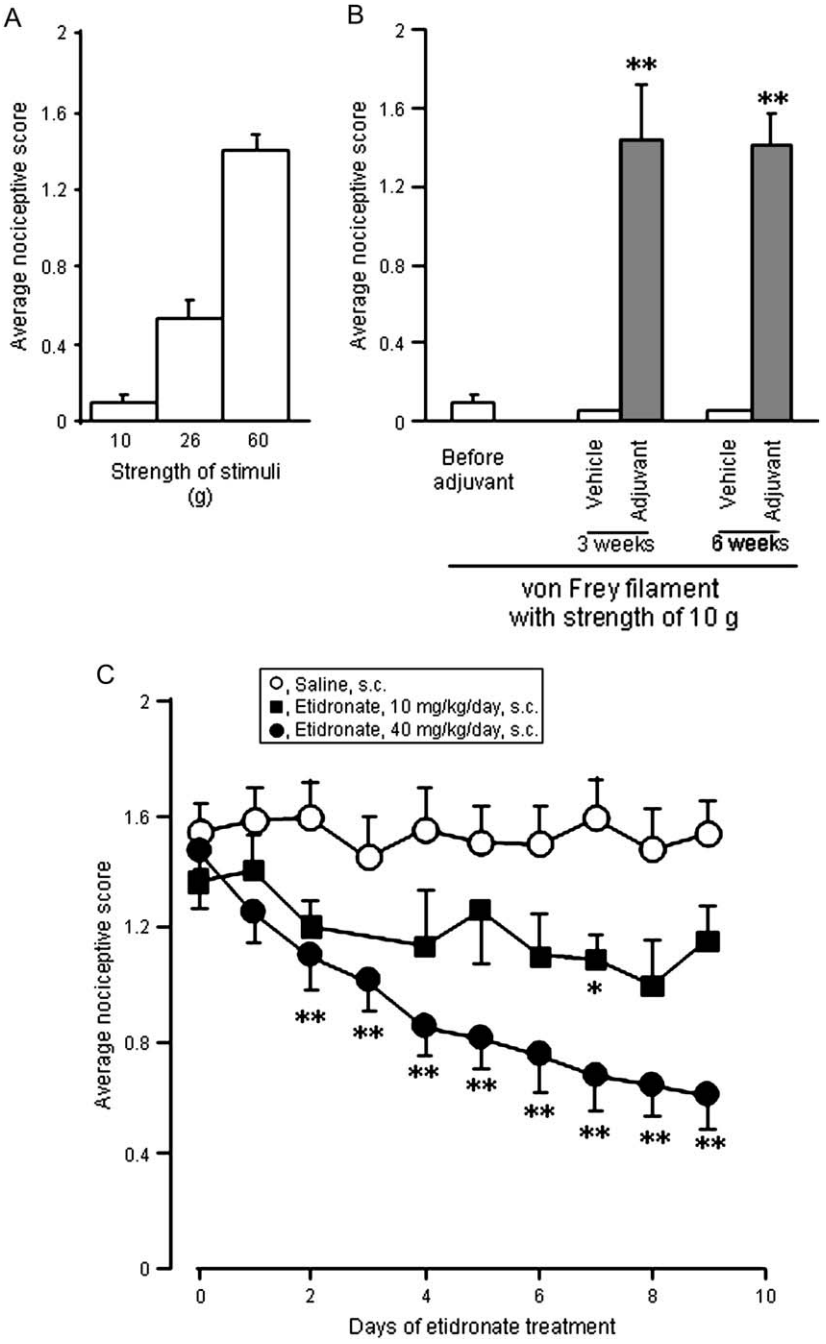


Fig. 1. Development of mechanical allodynia and its reversal by repeated administration of etidronate in rats treated with adjuvant. (A) Nociceptive scores in response to stimulation with distinct von Frey hairs in naïve rats. $n = 26$. (B) Increased nociceptive scores in response to 10-g filament in the adjuvant arthritic rats. $n = 26$ (before adjuvant) or 4–6 (after adjuvant or vehicle). $^{**}P < 0.01$ vs. vehicle. (C) Suppression of the adjuvant-evoked allodynia by repeated daily s.c. administration of etidronate in the rats. Etidronate was administered s.c. repeatedly once daily approximately 3 weeks after adjuvant challenge, and nociceptive assay was performed before administration of etidronate on each day. $n = 6$–16. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. saline.

rat dorsal root ganglia (DRG) using the reverse transcription-polymerase chain reaction (RT-PCR) technique. Under urethane (1.5 g/kg, i.p.) anesthesia, the rat was perfused transcardially with 100 ml of cold saline. The L4–L5 DRG were isolated and homogenized in TRIzol solution (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was extracted and reverse-transcribed at 42 °C for 50 min, followed by PCR amplification using the RNA LA PCR kit (AMV) version 1.1 (Takara, Kyoto, Japan). Amplification was allowed to proceed for 35 cycles (a cycle: denaturation at 94 °C for 30 s; 94; reannealing at 55 °C for 30 s; primer extension at 72 °C for 1 min). PCR primers employed were: 5′-AG GAAGATGCTGGCCAGG-3′ (sense) and 5′-AACCGTGATGGCCAGAGG-3′ (antisense), amplifying 480 bp fragments, for Kir6.1; 5′-GCCATGCTGTCCC GAAAA-3′ (sense) and 5′-GATGCAGCCCAGCATGAT-3′ (antisense), amplifying 504 bp fragments, for Kir6.2; 5′-TCCCTTCAATAAGCAACGGTAC-3′ (sense) and 5′-CATCCTCATCCAGCAGAAGGCC-3′ (antisense), yielding 539 bp products, for SUR1; 5′-GTTCTGCCTGGCCAGGGCC-3′ (sense) and 5′-GTCTACTTGTTGGTCATCACCAAA-3′ (antisense), yielding 285 bp products, for SUR2A; 5′-GTTCTGCCTGGCCAGGGCC-3′ (sense) and 5′-CCTA CAGGGAGTGTCCCTCAGACC-3′ (antisense), producing 450 bp fragments, for SUR2B (Cao et al., 2002); 5′-ACCACAGTCCATCAC-3′ (sense) and 5′-TCCACCACCTGTTGCTGTA-3′ (antisense), amplifying 452 bp fragments,

for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Kawabata et al., 2001). PCR products were verified by electrophoresis on 2% agarose gels and visualized under UV with ethidium bromide.

2.7. Drugs

Etidronate and alendronate were gifts from Sumitomo Pharmaceuticals Co., Ltd (Osaka, Japan). Glibenclamide and tolbutamide were purchased from Sigma (St. Louis, MO), and indomethacin was from Wako Pure Chem. (Osaka, Japan). Heat-killed *Mycobacterium butyricum* was obtained from Difco Laboratories (Detroit, MI).

2.8. Data analysis

Data are expressed as means with s.e.mean. Statistical evaluation was performed by Student's *t*-test for two-group comparisons or by ANOVA followed by Tukey's test for multiple comparisons, and differences were considered statistically significant at the level of $P < 0.05$.

3. Results

3.1. Antiallodynic activity of repeated administration of etidronate, a non-aminobisphosphonate, in rats

In naïve rats, neither single nor 10-time repeated daily s.c. administration of etidronate at 40 mg/kg altered the nociceptive scores in response to stimulation with von Frey filament with strength of 10, 26 or 60 g (data not shown). In the rats with adjuvant arthritis, etidronate, when administered s.c. repeatedly at 10 or 40 mg/kg/day, gradually suppressed the adjuvant-induced allodynia, as assessed by 10-g von Frey hair (Fig. 1C), although its single administration at the same doses did not affect the allodynia within 24 h (Fig. 1C; data not shown). The antiallodynic effect of daily treatment with etidronate became maximal on day 9, showing 60% or more reduction in the average nociceptive score (Fig. 1C).

3.2. Effect of systemic administration of glibenclamide on the antiallodynic effect of repeated doses of etidronate in adjuvant arthritic rats

Glibenclamide, an inhibitor of K^+_{ATP} channels, when administered s.c. at 10 mg/kg, almost completely reversed the antiallodynic effect of 10-time repeated doses of etidronate at 40 mg/kg/day in the arthritic rats (Figs. 2A,B). In the adjuvant-arthritic rats that received only vehicle (saline) for etidronate, systemic glibenclamide did not modify the adjuvant-evoked allodynia (Fig. 2C). It is also of note that systemic glibenclamide itself did not cause allodynia in naïve rats (Fig. 2D). Collectively, it is considered that glibenclamide specifically blocked the antiallodynic effect of etidronate.

3.3. Effect of i.pl. administration of glibenclamide and tolbutamide on the antiallodynic effect of repeated doses of etidronate in adjuvant arthritic rats

The decreased nociceptive score after 10-time repeated doses of etidronate at 40 mg/kg/day was reversed by i.pl. administration of glibenclamide at 160 μg/paw in the adjuvant arthritic rats, which, at 1 h, nearly reached a level equivalent to the nociceptive score before administration of etidronate (Figs. 3A,B). In contrast, i.pl. glibenclamide at the same dose did not modify the extent of the adjuvant-evoked allodynia in saline-treated arthritic animals (Fig. 3C). Tolbutamide, another inhibitor of K^+_{ATP} channels, administered i.pl. at 160 μg/paw, also abolished the antiallodynic effect of repeated doses of etidronate in the arthritic rats (Fig. 3D).

3.4. Antiallodynic activity of repeated administration of alendronate, an aminobisphosphonate, in adjuvant arthritic rats and its dependence on K^+_{ATP} channels

Like the non-aminobisphosphonate etidronate, the aminobisphosphonate alendronate, administered s.c. repeatedly at 1 mg/kg, gradually suppressed the adjuvant-induced allodynia (Fig. 4A). The time-course of the antiallodynic effect of alendronate at 1 mg/kg was similar to that of etidronate at 40 mg/kg/day (Fig. 4A). The antiallodynic effect of repeated doses of alendronate at 1 mg/kg was also strongly attenuated by i.pl. administration of glibenclamide at 160 μg/paw (Fig. 4B).

3.5. Effect of indomethacin on the adjuvant-evoked allodynia in rats and its independence of K^+_{ATP} channels

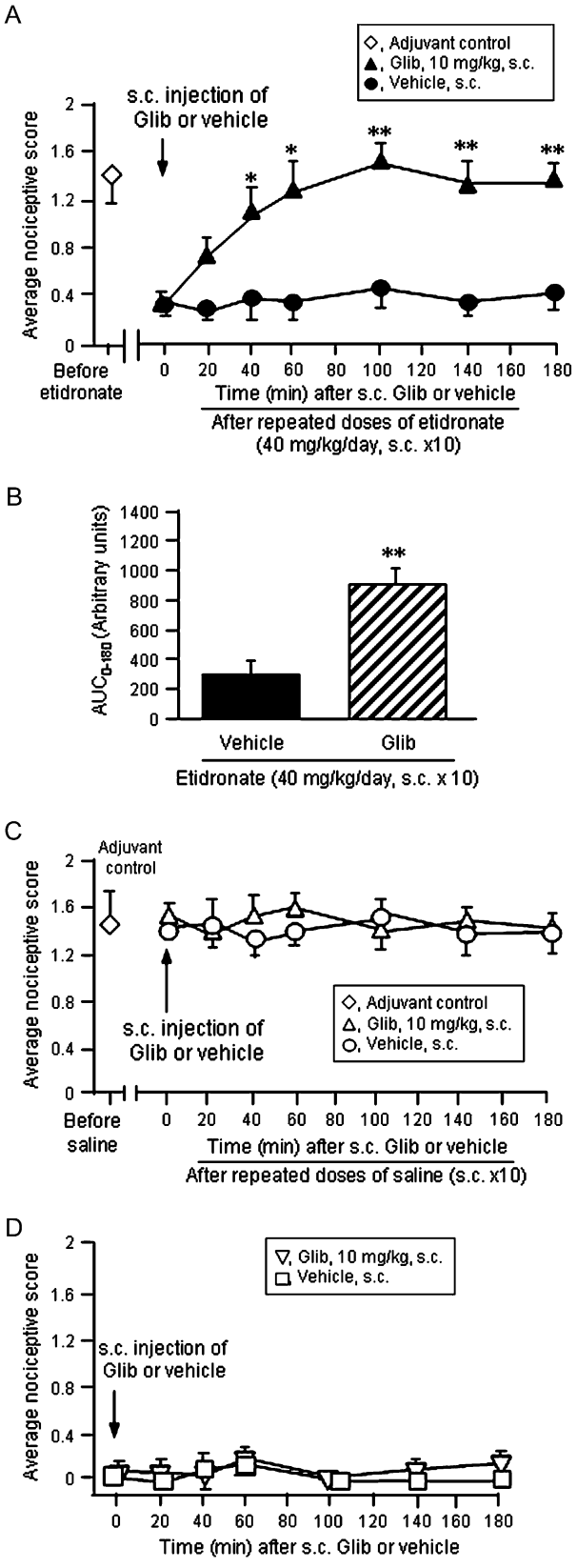
A single dose of s.c. indomethacin at 5 mg/kg caused only slight reduction in the nociceptive score in response to 10-g von Frey hair in the rats with adjuvant-evoked allodynia (Fig. 5A). However, indomethacin, administered s.c. repeatedly at the same dose, 10 times in total, strongly suppressed the adjuvant-induced allodynia (Fig. 5B). Glibenclamide, given i.pl. at 160 μg/paw, failed to inhibit the effect of repeated doses of indomethacin on the adjuvant-induced allodynia in the rats (Figs. 5B,C).

3.6. Effect of etidronate and alendronate on the hindpaw edema in rats with adjuvant-induced arthritis

Treatment of the right hindpaw with adjuvant caused dramatic increases in the thickness of both ipsilateral and contralateral hindpaws after 3–6 weeks (Fig. 6A). Repeated administration of etidronate at 40 mg/kg/day and alendronate at 1 mg/kg/day only slightly attenuated the increased thickness of either hindpaw 3 weeks after adjuvant challenge in the rats (Fig. 6B).

3.7. Detection of mRNAs for K^+_{ATP} channel subunits in rat DRG

Finally, we analyzed if rat DRG neurons would express mRNAs for K^+_{ATP} channel subunits including Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B. RT-PCR analyses show abundant expression of mRNAs for Kir6.1 and any types of SUR subunits, while mRNA for Kir6.2 was poor in the DRG (Fig. 7). Neither i.pl. administration of adjuvant nor s.c. administration of etidronate clearly affected mRNA levels for



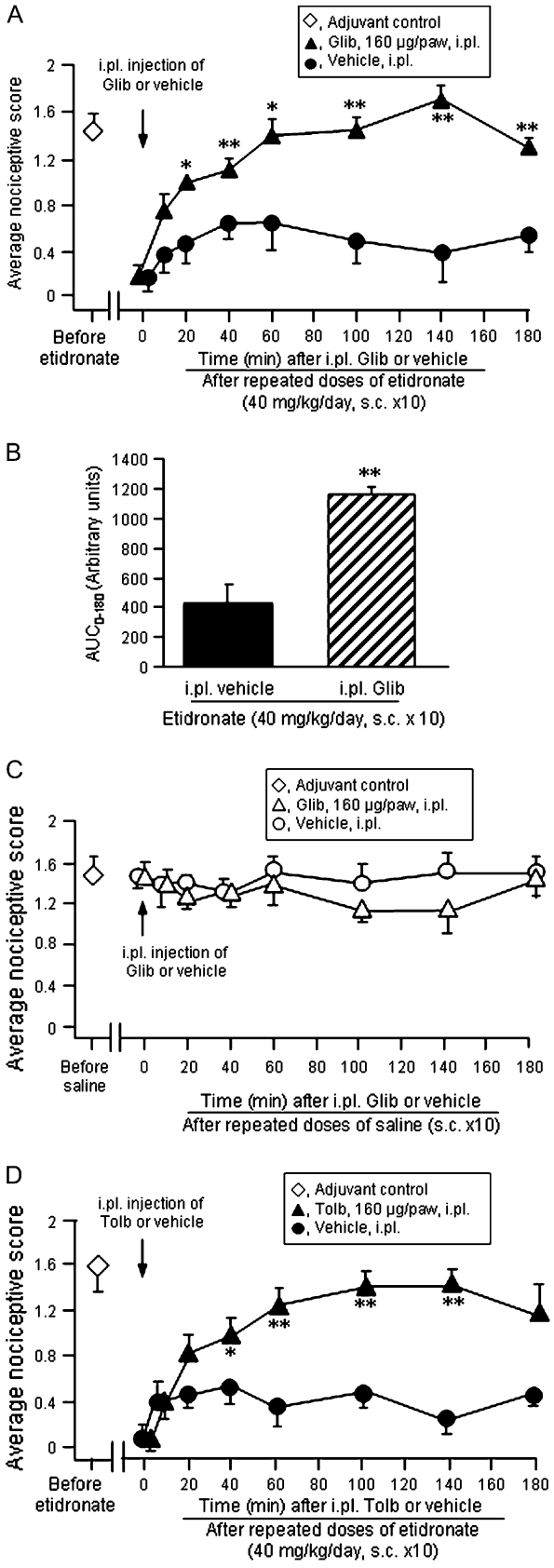
any K^+_{ATP} channel subunits in contralateral L5-L6 DRG in rats (Fig. 7).

4. Discussion

The present study demonstrates that etidronate as well as alendronate, administered repeatedly, exhibits antiallodynic activity in rats with adjuvant-induced arthritis, although their single administration had no effect. Our data also show, for the first time to our best knowledge, that the antiallodynic effects of the bisphosphonates can be reversed by glibenclamide and/or tolbutamide that unaffected the effect of indomethacin, implying involvement of K^+_{ATP} channels in the underlying mechanisms.

There is evidence from animal experiments that systemic or central administration of some bisphosphonates such as pamidronate and clodronate produce prompt and potent antinociception in naïve mice, whereas the antinociceptive activity of etidronate and alendronate is poor (Bonabello et al., 2001). However, such acute antinociceptive effects of single doses of the bisphosphonates shown in mice are not considered to be directly related to the potent antiallodynic activity of repeated doses of etidronate and alendronate in the present study, considering the delayed onset of effect in the arthritic rats (see Figs. 1C and 4A) and lack of effect in naïve rats (data not shown). Our present evidence might be, rather, relevant to the clinically detectable analgesic property of bisphosphonates (Adami, 1997; Adami et al., 1997; Forys, 1991; Kubalek et al., 2001; Storm et al., 1990; Varenna et al., 2000). The slight but significant inhibitory effects of etidronate and alendronate on the adjuvant-induced hindpaw edema (see Fig. 6) are consistent with the recent report (Harada et al., 2004), in which the bisphosphonates, administered daily for 3 weeks immediately after adjuvant challenge, moderately reduced the paw swelling. The relatively weak anti-inflammatory effect of the bisphosphonates in the present study may be due to the distinct administration schedule, in which repeated administration of the bisphosphonates was started after establishment of adjuvant-induced arthritis (3 weeks after adjuvant challenge). Such weak anti-inflammatory effects of those bisphosphonates do not fully explain their potent antiallodynic activity in the arthritic rats; i.e., the latter effects of the bisphosphonates are not necessarily secondary to the former effects, predicting involvement of alternative mechanisms including direct or

Fig. 2. Effect of systemic administration of glibenclamide (Glib) on the antiallodynic effect of repeated doses of etidronate in the rats with adjuvant-induced arthritis. (A) and (B) Effect of s.c. glibenclamide at 10 mg/kg on the decreased nociceptive scores in response to 10-g von Frey hair following 10-time repeated s.c. administration of etidronate at 40 mg/kg/day in the adjuvant arthritic rats. Inhibition experiments were conducted on the day after the final dose of etidronate. The time course of nociceptive scores (A) and the area under the curve for 180 min (AUC_{0-180}) (B) after s.c. glibenclamide. $n = 5$. $^*P < 0.05$, $^{**}P < 0.01$, vs. vehicle for glibenclamide. (C) Lack of effect of s.c. glibenclamide on the nociceptive scores in response to 10-g von Frey hair in the arthritic rats that received repeated daily s.c. administration of saline. $n = 5$. (D) Lack of effect of s.c. glibenclamide on the nociceptive scores in response to 10-g von Frey hair in naive rats. $n = 5$.



indirect functional modulation of afferent nerves. It has been shown that the anti-resorptive effect of alendronate in rats with osteoporosis is approximately 1000-fold stronger than etidronate (Lin, 1996; Sahni et al., 1993). Nonetheless, the anti-allodynic activity of etidronate at 40 mg/kg/day was even greater than alendronate at 1 mg/kg/day (see Fig. 4A), indicating that the relative potency of alendronate as an antiallodynic agent to etidronate should be less than 40. This value almost corresponds to their relative potency (38–42) as anti-inflammatory agents in arthritic rats as described elsewhere (Harada et al., 2004). It has been described that etidronate in a dose range of 5–10 mg/kg/day dose-dependently prevents the decrease in bone mineral density (BMD) in the proximal tibia of the arthritic rats, while alendronate does in a range of 0.001–0.1 mg/kg/day (Harada et al., 2004). Of note is that etidronate even at 10 mg/kg/day produced weak but significant antiallodynic effect (see Fig. 1C). Thus, the effective dose range of etidronate as an antiallodynic agent appears to be much closer to that for its anti-resorptive effect, compared with alendronate, predicting that etidronate, but not alendronate, even at the present clinical therapeutic doses used for bone diseases, might produce analgesia in arthritic patients. In addition, because of abdominal side effects reported in clinical trials (de Groen et al., 1996), it might be impossible to increase oral doses of alendronate for treatment of arthritis patients. Pharmacokinetic profiles of repeated doses of the bisphosphonates in arthritic animals have yet to be investigated, although their general pharmacokinetic properties have already been well-described (Lin, 1996).

K^+_{ATP} channels are now known to be distributed in a wide variety of tissues/cells including neurons (Yokoshiki et al., 1998). Our findings that both systemic and local (i.p.l.) administration of K^+_{ATP} channel inhibitors specifically blocked the antiallodynic effects of repeated doses of etidronate or alendronate, strongly suggest that both bisphosphonates would modulate functions of peripheral K^+_{ATP} channels, leading to the antiallodynic activity. Our data from RT-PCR analysis of rat DRG also imply abundant expression of mRNAs for K^+_{ATP} channel subunits, Kir6.1, SUR1, SUR2A and SUR2B, but a low level of expression of Kir6.2 mRNA, although their identification at protein levels has yet to be done. There is electrophysiological

Fig. 3. Effect of i.pl. administration of glibenclamide (Glib) or tolbutamide (Tolb) on the antiallodynic effect of repeated doses of etidronate in the rats with adjuvant-induced arthritis. (A) and (B) Effect of i.pl. glibenclamide at 160 μg/paw on the decreased nociceptive scores in response to 10-g von Frey hair following 10-time repeated s.c. administration of etidronate at 40 mg/kg/day in the adjuvant arthritic rats. The time course (A) and the area under the curve for 180 min (AUC_{0-180}) (B) after i.pl. glibenclamide. $n = 4$. $^*P < 0.05$, $^{**}P < 0.01$, vs. vehicle for glibenclamide. (C) Lack of effect of i.pl. glibenclamide on the nociceptive scores in response to 10-g von Frey hair in the arthritic rats that had received repeated daily s.c. administration of saline. $n = 4$. (D) Effect of i.pl. tolbutamide at 160 μg/paw on the decreased nociceptive scores in response to 10-g von Frey hair following 10-time repeated s.c. administration of etidronate at 40 mg/kg/day in the adjuvant arthritic rats. $n = 4$. $^*P < 0.05$, $^{**}P < 0.01$, vs. vehicle for tolbutamide. All inhibition experiments were conducted on the day after the final dose of etidronate or saline.

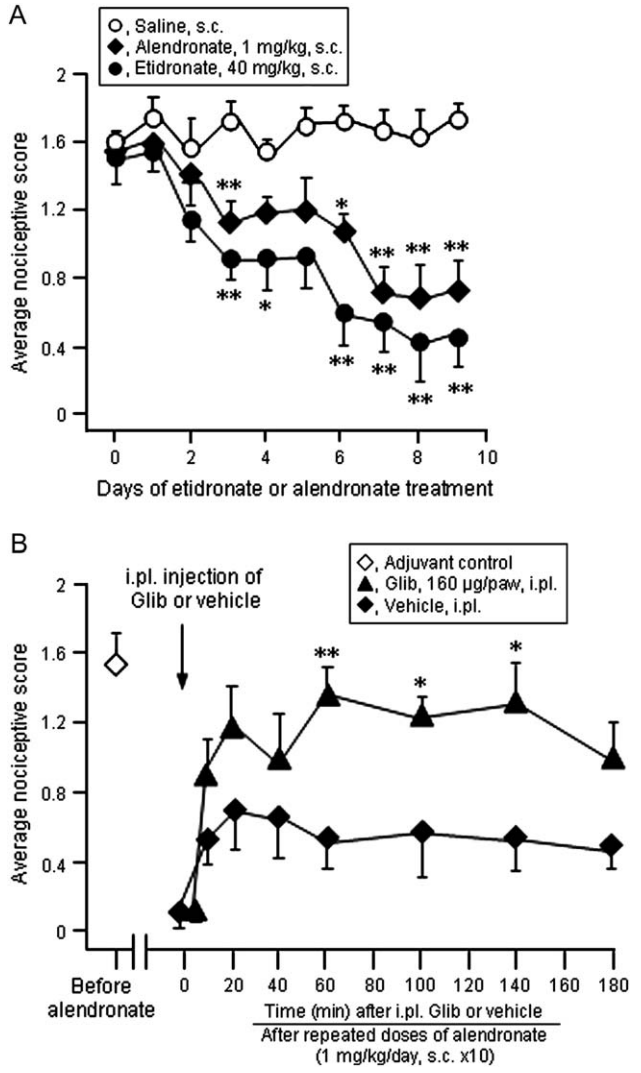


Fig. 4. Antiallodynic effect of repeated administration of alendronate and its reversal by glibenclamide (Glib) in the rats with adjuvant-induced arthritis. (A) Suppression of the adjuvant-evoked allodynia by daily repeated s.c. administration of alendronate at 1 mg/kg or etidronate at 40 mg/kg in the rats. Nociceptive assay was performed before drug administration on each day. $n = 6-7$. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. saline. (B) Effect of i.pl. glibenclamide at 160 μg/paw on the decreased nociceptive scores in response to 10-g von Frey hair following 10-time repeated s.c. administration of alendronate at 10 mg/kg/day in the adjuvant arthritic rats. Inhibition experiments were conducted on the day after the final dose of alendronate. $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. vehicle for glibenclamide.

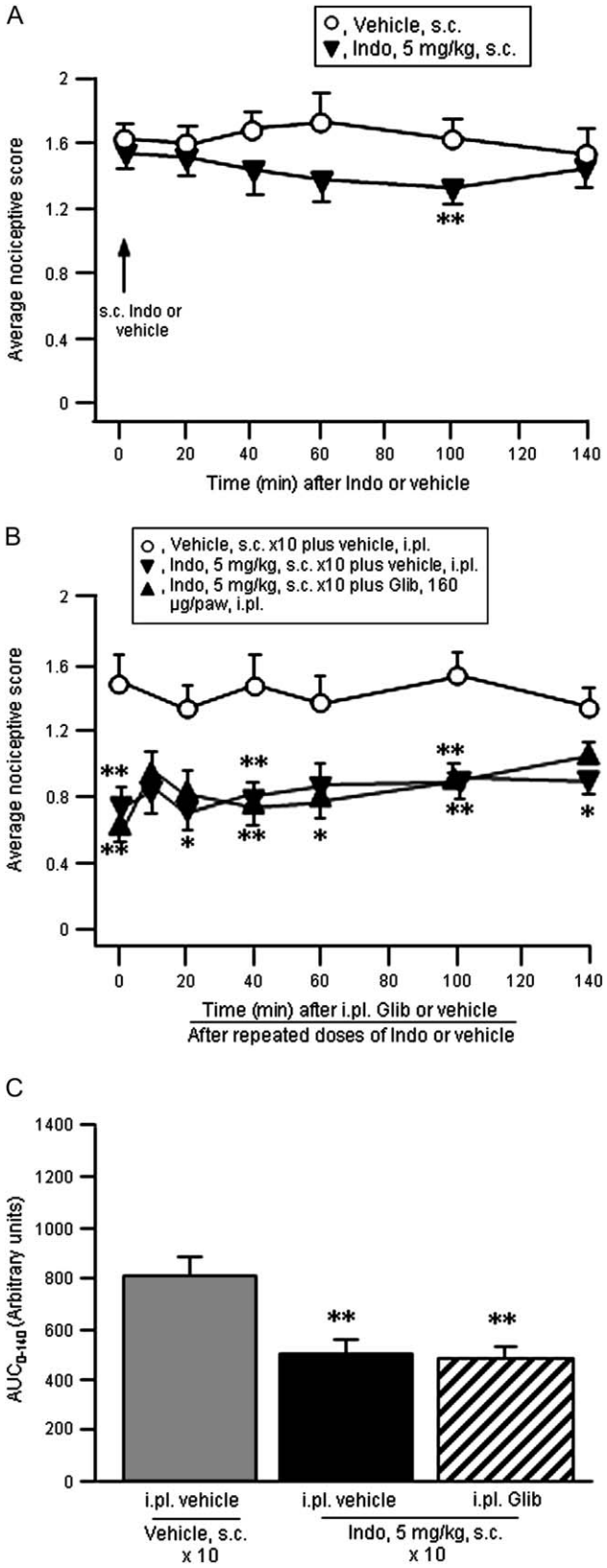


Fig. 5. Effect of single or repeated administration of indomethacin (Indo) on the adjuvant-evoked allodynia and its resistance to glibenclamide (Glib) in the rats. (A) Time-related effect of single s.c. administration of indomethacin at 5 mg/kg on the increased nociceptive scores in response to 10-g von Frey hair in the arthritic rats. $n = 6-7$. $^{**}P < 0.01$, vs. vehicle for indomethacin. (B) and (C) Glibenclamide-resistant effect of repeated administration of indomethacin on the adjuvant-induced allodynia in the rats. Inhibition experiments were conducted on the day after the final dose of indomethacin. Time-course of nociceptive scores (B) and the area under the curve for 140 min (AUC_{0-140}) (C) after i.pl. glibenclamide in the arthritic rats that had received repeated doses of s.c. indomethacin for 10 days. $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. vehicle for indomethacin plus vehicle for glibenclamide.

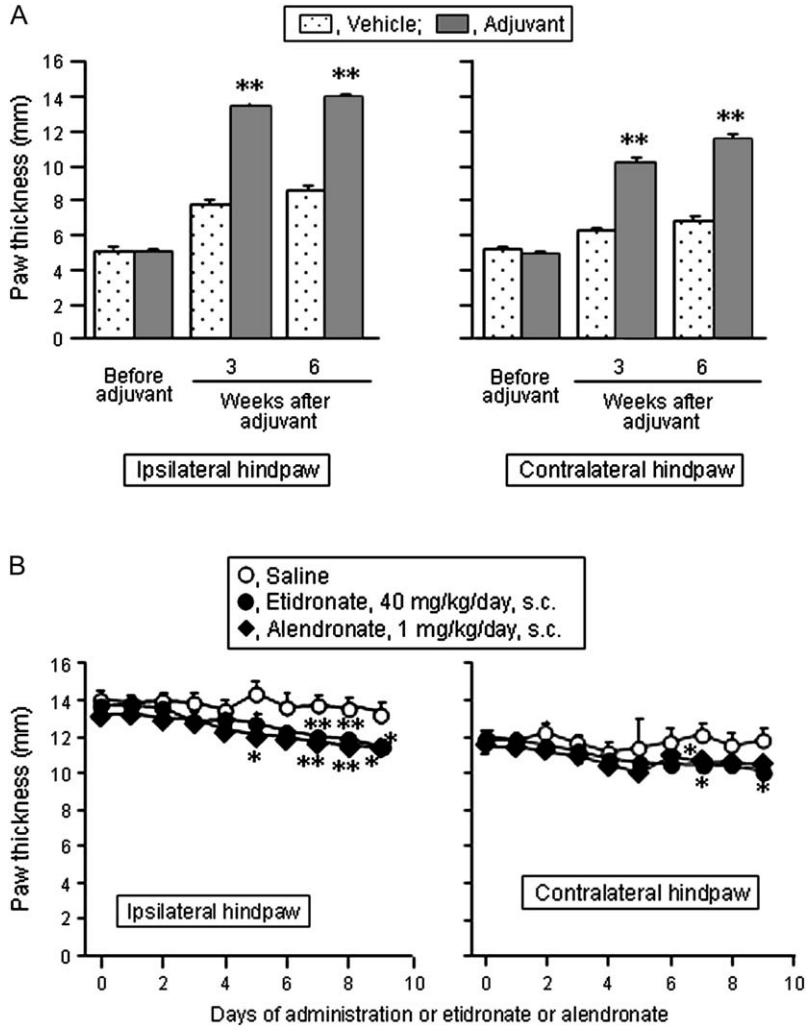


Fig. 6. Development of edema and its inhibition by repeated administration of bisphosphonates in the rats treated with adjuvant. (A) Ipsilateral and contralateral paw thickness in the rats that had received i.pl. administration of adjuvant in the right hindpaw. $n = 4$ (vehicle) and 11–20 (adjuvant). $^{**}P < 0.01$, vs. vehicle. (B) Effect of repeated daily s.c. administration of etidronate at 40 mg/kg/day or alendronate at 1 mg/kg/day on the increased thickness of ipsilateral and contralateral hindpaws in adjuvant arthritic rats. $n = 6$–7. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. saline.

evidence that K^+_{ATP} channels are expressed by the primary afferent neurons, particularly nociceptor neurons (Ristoiu et al., 2002; Sarantopoulos et al., 2003). A number of pharmacological studies have provided functional evidence for involvement of K^+_{ATP} channels in the peripheral analgesic mechanisms (Pacheco and Duarte, 2005; Picolo et al., 2003; Sachs et al., 2004). It is unlikely that the bisphosphonates directly activate K^+_{ATP} channels in the primary afferent nerves, since their antiallodynic effects slowly developed only after their repeated administration for 2–10 days (see Figs. 1C and 4A). Considering the previous evidence that activation of the arginine/NO/cyclic GMP/protein kinase G pathway might cause peripheral analgesic blockade of hypernociception by activating K^+_{ATP} channels (Sachs et al., 2004), it could be speculated that the bisphosphonates might enhance the activity of the arginine/NO/cyclic GMP/protein kinase G pathway, resulting in delayed antiallodynic effect. Our RT-PCR analyses suggest that neither adjuvant treatment nor administration of bisphosphonates clearly alter levels of mRNAs of K^+_{ATP} channels, although this has yet to be further examined using more quantitative techniques such as real time PCR and Western blotting.

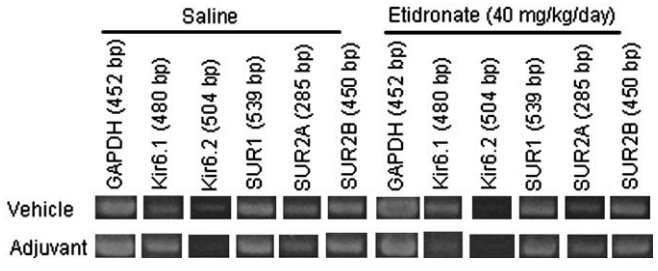


Fig. 7. RT-PCR analysis of mRNAs for K^+_{ATP} channel subunits, Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B, in contralateral dorsal root ganglia (DRG) of rats with or without adjuvant arthritis that received vehicle or etidronate. Etidronate or vehicle was administered s.c. repeatedly once daily for 10 days, from 3 weeks after adjuvant challenge. Data show the results from RT-PCR analyses of contralateral L4–L5 DRG collected from 3 rats.

The present study thus provides novel evidence that both non-aminobisphosphonates and aminobisphosphonates

attenuate arthritis-related allodynia by modulating K^+_{ATP} channels, suggesting potential therapeutic benefits of these agents.

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