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Potent inhibition by trivalent cations of ATP-gated channels

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Abstract

The effects of La^{3+} and other trivalent cations on ATP-gated channels (P_{2X} purinoceptor/channels) were investigated using rat pheochromocytoma PC12 cells and *Xenopus* oocytes expressing these channels. La^{3+}, Gd^{3+}, Ce^{3+} and Nd^{3+} (30–300 μM) inhibited an inward current activated by 30 μM ATP in PC12 cells. The concentration–response curve for the ATP-activated current was shifted by La^{3+} or Gd^{3+} toward a higher concentration range, and the slope of the curve became steeper, suggesting the inhibition is non-competitive. La^{3+} or Gd^{3+} did not affect the current component that was slowly activated upon hyperpolarization, and selectively inhibited the remaining 'voltage-independent' component. La^{3+} and Gd^{3+} also inhibited currents mediated through P_{2X1} and P_{2X2} purinoceptors expressed in *Xenopus* oocytes. The results suggest that La^{3+} and other trivalent cations inhibit P_{2X} purinoceptors at low concentrations. The inhibition may at least partly be attributed to an allosteric inhibition.

Keywords: ATP; Purinoceptor P_{2X}; Trivalent cation; PC12 cell; *Xenopus* oocyte

1. Introduction

Extracellular ATP activates non-selective cation channels, and these ATP-gated channels are now classified into P_{2X} purinoceptors (Abbracchio and Burnstock, 1994). cDNAs encoding P_{2X} purinoceptors have been cloned, and it has been shown that the P_{2X} purinoceptor family consist of genetically different multiple subclasses (for review, see North, 1996). Physiological roles of P_{2X} purinoceptors, such as in signal transduction in neurons and smooth muscle cells in response to excitatory neurotransmission by ATP, have also been suggested (for review, see Dubyak and El-Moatassim, 1993).

Various compounds are known to modulate P_{2X} purinoceptors. Interestingly, most of these modulators exhibit facilitatory effects on macroscopic currents through P_{2X} purinoceptor/channels. For endogenous substances, dopamine (Inoue et al., 1992; Nakazawa et al., 1993), 5-hydroxytryptamine (Nakazawa et al., 1994c), adenosine (Inoue et al., 1994b) and Zn^{2+} (Clouse et al., 1993; Li et al., 1993; Koizumi et al., 1995) enhance ionic currents activated by ATP in peripheral neuronal cells. Among exogenous substances, Cd^{2+} (Ikeda et al., 1996), capsaicin (Nakazawa et al., 1994b) and alkyl-*p*-hydroxybenzoic acid (Inoue et al., 1994a) enhance the ATP-activated current. Single-channel currents through P_{2X} purinoceptors are, however, reduced by divalent cations including Ca^{2+} and Cd^{2+} (Nakazawa and Hess, 1994). Dopamine, 5-hydroxytryptamine, Zn^{2+} and Cd^{2+} also facilitate P_{2X} purinoceptors expressed from the cloned cDNA in *Xenopus* oocytes (Nakazawa and Ohno, 1996).

For the present study, we investigated the effects of La^{3+} and other trivalent cations on the ionic current permeating through P_{2X} purinoceptors in PC12 cells and that through P_{2X1} or P_{2X2} purinoceptor/channels expressed in *Xenopus* oocytes. We found that these trivalent cations inhibited the ATP-activated current at low concentrations.

2. Materials and methods

2.1. PC12 cells

PC12 cells (passage 55–70) were cultured according to Inoue and Kenimer (1988). Cells were plated on collagen-coated coverslips placed on the bottom of 35-mm polystyrene dishes. Current recordings were made with conventional whole-cell voltage-clamp methods (Hamill et al., 1981) under the conditions described elsewhere

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(Nakazawa et al., 1990, 1994c). The cells were bathed in an extracellular solution containing (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 10, D-glucose 11.1 (pH was adjusted to 7.4 with NaOH). Tip resistances of heat-polished patch pipettes ranged between 3 and 5 MΩ when the pipettes were filled with an intracellular solution containing (in mM) CsCl 150, HEPES 10, ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 5 (pH 7.3 with CsOH). Application of ATP was made by superfusion, and each application was separated by 1 min. Trivalent cations were applied for 10–20 s before and during the application of ATP. Experiments were performed at room temperature (about 25°C). Electrical signals were recorded with a patch-clamp amplifier (Nihon Kohden CEZ-2400, Tokyo Japan), filtered at 5 kHz and stored on magnetic tape for later analysis.

2.2. *Xenopus* oocytes expressing P_{2X} purinoceptor / channels

P_{2X} purinoceptors were expressed in *Xenopus* oocytes and current recordings were made from the oocytes according to our previous reports (Nakazawa et al., 1994a; Nakazawa and Ohno, 1996). cDNA encoding P_{2X1} purinoceptor (Valera et al., 1994; the EMBL submission X80477) and that encoding P_{2X2} purinoceptor (Brake et al., 1994; the Genbank entry U14414) were kindly supplied by Dr. G. Buell of Glaxo Institute for Molecular Biology and Dr. T. Brake of the University of California, San Francisco, respectively. Sense fragments of RNA were transcribed from the DNA and injected into defolliculated *Xenopus* oocytes. The oocytes were incubated at 18°C in ND96 solution containing (mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.5 with NaOH) supplemented with 0.01% gentamycin for 3–6 days. Membrane currents were measured using two microelectrode voltage-clamp methods at room temperature (25–27°C). Oocytes were placed in an experimental chamber filled with ND96 solution. ATP and trivalent cations were applied by superfusion. The application of ATP was separated by 1 min for P_{2X2} purinoceptors, and the interval was increased to 3 min for P_{2X1} purinoceptors because these channels were more readily desensitized.

2.3. Drugs

ATP (adenosine 5′-triphosphate disodium salt) was purchased from Sigma (St. Louis, MO, USA). Trivalent cations used were chloride salts purchased from Wako Pure (Osaka, Japan). All the other compounds were of a reagent grade.

2.4. Statistical analysis

Statistical analysis was done by using the paired *t*-test or Duncan's multiple comparison.

3. Results

3.1. Block by trivalent cations of ATP-activated current in PC12 cells

Fig. 1A illustrates an inward current activated by 30 μM ATP in a PC12 cell. La^{3+} (10 μM) inhibited the ATP-activated current by about 40%, and the current readily recovered upon washout of La^{3+}. The inhibition was concentration dependent, and IC_{50} was about 10 μM (Fig. 1B). A similar reversible inhibition was also observed with Gd^{3+}, Ce^{3+} and Nd^{3+}, and the sensitivities of the ATP-activated current to these trivalent cations as well as La^{3+} were almost identical (Fig. 1B).

The magnitude of the current block by 30 μM La^{3+} was attenuated when the concentration of ATP was increased from 30 to 300 μM (Fig. 2A); the current remaining after the block produced by 30 μM La^{3+} was 74.9 ± 6.1% with ATP 300 μM ($n = 10$), which was significantly larger than the value with ATP 30 μM (4.3 ± 1.1%, $n = 12$; $P < 0.01$). The attenuation suggests that the current block depends on the concentration of ATP. To

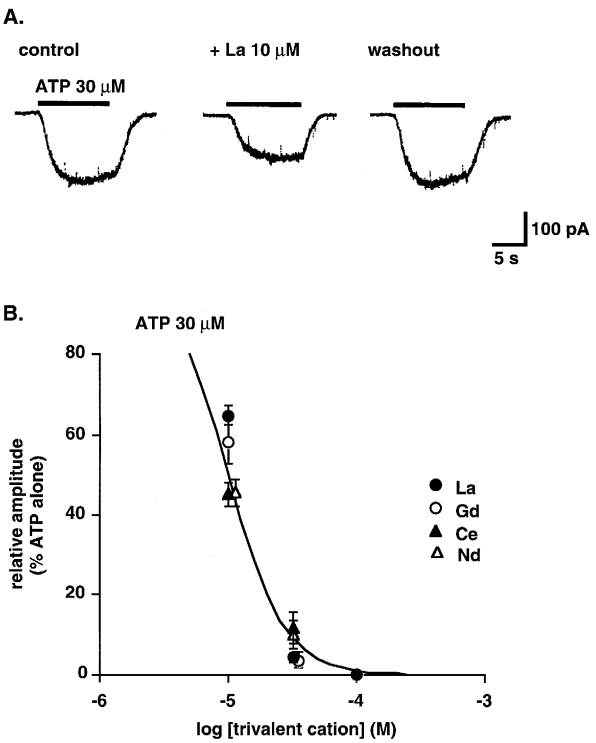

Fig. 1. Effects of trivalent cations on an inward current activated by 30 μM ATP in PC12 cells. The cells were held at −60 mV. (A) Reversible inhibition by 10 μM La^{3+} of the ATP-activated current. The current responses with three sequential applications of ATP to the same cell are shown. (B) Concentration dependence of the current inhibition by various trivalent cations. The current amplitude in the presence of La^{3+} (filled circles), Gd^{3+} (open circles), Ce^{3+} (filled triangles) or Nd^{3+} (open triangles) was normalized to that before the trivalent cation application. A smooth curve was drawn assuming an EC_{50} of 10 μM and a Hill coefficient of 2.0. Each symbol and bar represent the mean and S.E.M. obtained from 4–6 cells tested.

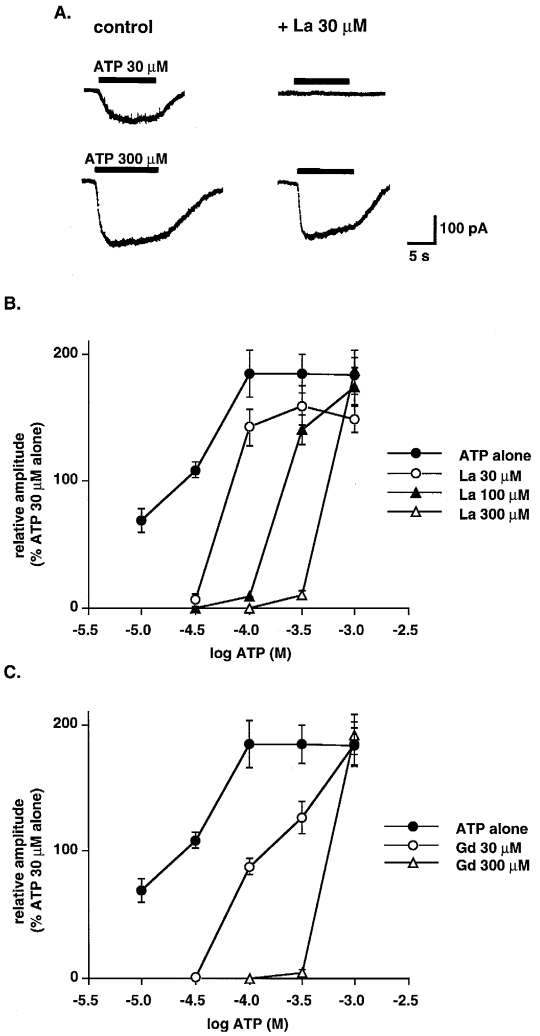

Fig. 2. (A) Attenuation by a higher ATP concentration of the current inhibition elicited by La^{3+}. A current activated by 30 μM ATP was abolished by 30 μM La^{3+} (upper traces) whereas that activated by 300 μM ATP was only slightly inhibited. Holding potential was −60 mV. (B,C) Concentration-response relationship for the ATP-activated current, and its shift by La^{3+} (B) or Gd^{3+} (C). The current amplitude at −60 mV obtained under various conditions was normalized to that obtained with 30 μM ATP in the absence of trivalent cations in individual cells. Each symbol represents the mean of the data obtained from 4–6 cells without trivalent cations (filled circles) or with 30 (open circles), 100 (filled triangles) or 300 μM trivalent cations (open triangles). Bars are S.E.M.

determine the relation between La^{3+} and the concentration of ATP, we obtained the concentration–response curves for the ATP-activated current in the absence and presence of La^{3+} (Fig. 2B,C). As a complete set of the data for overall concentration–response curves were difficult to obtain from individual cells, an inward current was elicited by 30 μM ATP in the absence of La^{3+} in each cell, and currents evoked under various conditions were normalized to this current. La^{3+} dose dependently shifted the concentration–response curve for the ATP-activated current toward a higher concentration range (Fig. 2B). In addition, the slope of the concentration–response curve became steeper in the presence of La^{3+}. In fact, the concentration–response curve with La^{3+} was so steep that a 3-fold increase in the ATP-concentration (e.g., increase from 100 to 300 μM in the presence of 100 μM La^{3+}) resulted in a dramatic shift from minimal current responses to almost maximal current responses. The concentration–response curve was also shifted by 30 and 300 μM Gd^{3+} (Fig. 2C), and, at 300 μM, the slope became also steeper.

3.2. Voltage dependence

Voltage dependence of the inhibition produced by trivalent cations of the ATP-activated current was examined by applying a stepwise pulse from +50 to −100 mV with an increment of −10 mV (Fig. 3). The ATP-activated current

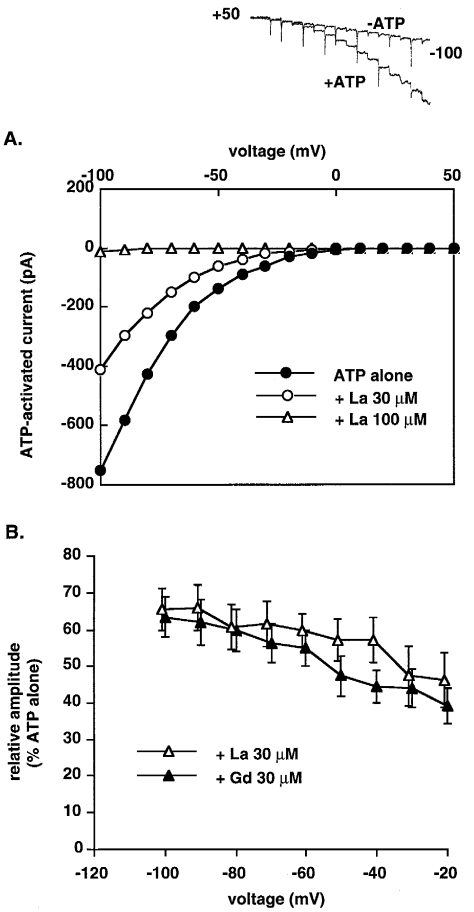

Fig. 3. Voltage dependence of the inhibition by trivalent cations of the ATP-activated current. The voltage dependence was determined with a stepwise pulse changing from +50 to −100 mV with an increment of −10 mV (50 ms for each step; see inset) in the absence and presence of trivalent cations. The concentration of ATP was 100 μM. (A) The current-voltage relation for the ATP-activated current. The relation obtained for a PC12 cell without (filled circles) or with 30 (open circles) or 100 μM La^{3+} (open triangles) is plotted. (B) The remaining current in the presence of 30 μM La^{3+} (open triangles) or Gd^{3+} (filled triangles). The current was measured as in (A) from 5 (La^{3+}) or 6 cells (Gd^{3+}), and the current amplitude with the trivalent cations was normalized to that with ATP alone for each potential. Each symbol and bar is the mean and S.E.M.

exhibited an inward rectification as previously reported (Nakazawa et al., 1990), and the outward current at positive potential was not resolved in the absence or presence of La^{3+} (Fig. 3A). For the inward current at potentials more negative than −20 mV, the current remaining after the block by 30 μM La^{3+} or Gd^{3+} was plotted against the potentials (Fig. 3B). The current was less remarkably inhibited at more negative potentials, and, for example, the remaining current at −100 mV was significantly larger

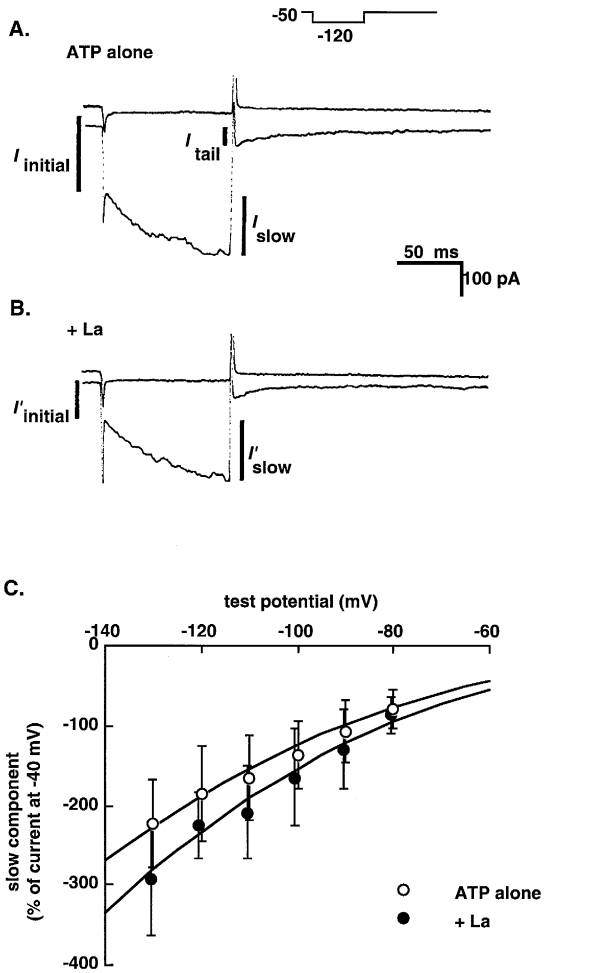

Fig. 4. (A,B) A slowly activated component (I_{slow}) of the ATP-evoked current in the absence (A) and presence of 30 μM La^{3+}. The concentration of ATP was 100 μM. The cell was held at −50 mV, and a 100 ms hyperpolarizing step to −120 mV was applied every 2 s. The current traces before and during the application of ATP are superimposed in each panel. La^{3+} largely inhibited the current at the beginning of the hyperpolarizing step ($I_{initial}$ vs. $I'_{initial}$) whereas it did not affect the slowly activating current (I_{slow} vs. I'_{slow}). A small transient inward current upon returning to the holding potential of −50 mV (tail current; I_{tail} in (A)) was not markedly inhibited by La^{3+} compared with $I_{initial}$ (see Table 1). (C) The voltage dependence of the slowly activating current. The cells were held at −40 mV and hyperpolarizing steps from −80 to −130 were repeatedly applied. The slowly activating component was defined as in (A) and (B). The amplitude of the slowly activating current was normalized to the absolute values of the amplitude of the ATP-evoked current at −40 mV. The concentration of ATP was 30 μM. The data are the mean and S.E.M. obtained from 4 cells in the absence (open circles) or presence of 30 μM La^{3+} (filled circles).

Table 1
Effects of La^{3+} on components of the ATP-activated current in PC12 cells

Current components	La^{3+} (30 μM)	Gd^{3+} (30 μM)
I_{-50}	45.3±6.7% (10)	57.8+2.7 (11)
$I_{-120,total}$	63.6±5.9 (10)	67.5+3.2 [a] (11)
$I_{initial}$	52.7±5.4 (10)	59.7±3.4 (11)
I_{slow}	95.3±8.5 [b] (10)	97.4±13.4 [a] (11)
I_{tail}	78.5±8.7 [b] (6)	83.9±10.0 (5)

I_{-50} and $I_{-120,total}$ are the ATP-activated current at −50 mV and the current at the end of a 100-ms step to −120 mV, respectively. Other current components were defined as in Fig. 4A,B. The amplitude of the components in the presence of La^{3+} or Gd^{3+} was normalized to that before trivalent cation application, and the values are given as means± S.E.M. Numbers of cells are shown in parentheses. Statistically significant difference from I_{-50} determined by Duncan's multiple comparison: [a] $P < 0.05$, [b] $P < 0.01$.

than that at −20 mV with La^{3+} (paired *t*-test, $P < 0.05$) or Gd^{3+} ($P < 0.001$).

The relief of the current block at negative potentials was further confirmed by applying a hyperpolarizing step to −120 mV from a holding potential of −50 mV (Fig. 4A,B; Table 1). Under this condition, the current at −120 mV, determined at the end of the hyperpolarizing pulse ($I_{-120,total}$), was also more weakly inhibited by La^{3+} or Gd^{3+} than that at −50 mV (Table 1), and the difference was statistically significant with Gd^{3+}. The current at −120 mV (Fig. 4A) exhibited a slowly activating component. A similar slowly activating component was reported in sympathetic neurons isolated from rat superior cervical ganglia (Nakazawa, 1994). This component may reflect a voltage-dependent gating of the ATP-activated channels. The inward tail current observed upon returning to −50 mV (Fig. 4A, I_{tail}) supported the existence of such voltage-dependent gating. We separated this slowly activated component (I_{slow}) from an initial instantaneous component ($I_{initial}$), and compared the magnitude of the inhibition for these components (Table 1). $I_{initial}$ at −120 mV was inhibited by La^{3+} or Gd^{3+} as strongly as the current at −50 mV (I_{-50}), whereas I_{slow} at −120 mV was scarcely inhibited. The results suggest that trivalent cations selectively inhibit the voltage-independent current component. In accord with this, I_{tail}, indicative of voltage-dependent gating, was also more weakly affected than I_{-50}.

The voltage dependence of the slowly activating component was determined in the absence and presence of La^{3+}. To determine the amplitude of this current component at various potentials during single trials of ATP, the cells were hyperpolarized from a holding potential of −40 mV to various potentials, and the slow component at these potentials was normalized to the ATP-activated current at

−40 mV. The relative amplitude of the slow component determined in this way was larger in the presence of La^{3+} than in the absence of La^{3+} because the current at −40 mV was largely inhibited by La^{3+} (Fig. 4C). The voltage dependence of the slow component was, however, not affected by La^{3+}.

3.3. ATP-activated current in Xenopus oocytes expressing P_{2X} purinoceptor / channels

Fig. 5A,B shows the current activated by ATP recorded from *Xenopus* oocytes expressing P_{2X1} purinoceptors or P_{2X2} purinoceptors. As P_{2X1} purinoceptors exhibit much higher sensitivity to ATP (Valera et al., 1994) than P_{2X2} purinoceptors (Brake et al., 1994), a lower concentration of ATP was used for P_{2X1} purinoceptors (100 nM) than for P_{2X2} purinoceptors (30 μM). La^{3+} inhibited the current mediated through P_{2X2} purinoceptors at concentrations similar to those required for the current inhibition in PC12 cells (see Fig. 1B). The current was blocked by Gd^{3+} at lower concentrations (Fig. 5A,C). La^{3+} and Gd^{3+} also inhibited the current permeating through P_{2X1} purinoceptors at higher concentrations compared with P_{2X2} purinoceptors (Fig. 5B,C).

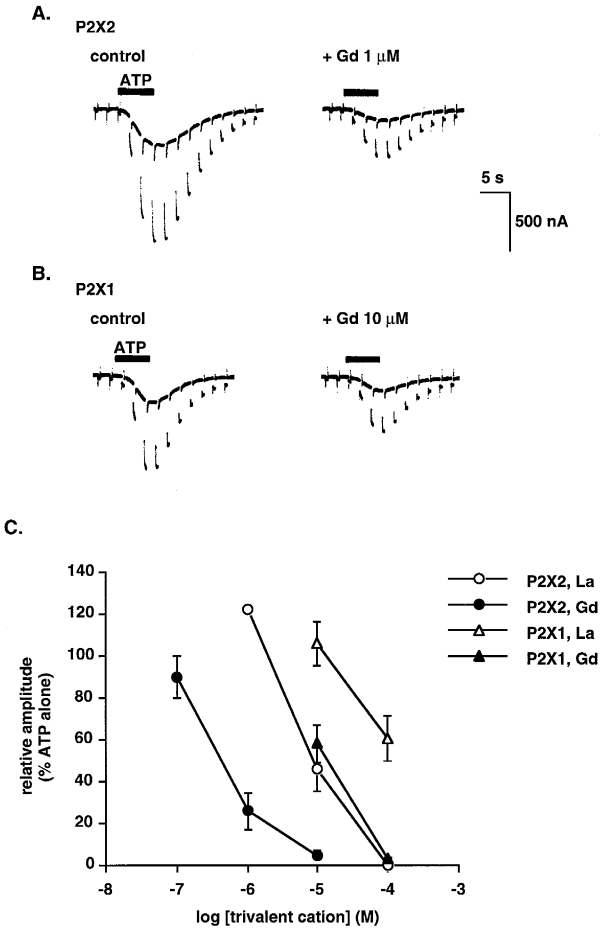

Fig. 5. Inhibition by trivalent cations of P_{2X} purinoceptors expressed in *Xenopus* oocytes. The oocytes were held at −50 mV and a 200 ms voltage step to −80 mV was applied every 2 s. The concentration of ATP for the current activation was 30 μM (P_{2X2} purinoceptors) or 100 nM (P_{2X1} purinoceptors), respectively. (A,B) Inhibition by Gd^{3+} of the current permeating through P_{2X2} (A) or P_{2X1} purinoceptors (B). Gd^{3+} largely blocked the current through P_{2X2} purinoceptors at 1 μM (A) whereas a higher concentration (10 μM) of Gd^{3+} was required for comparable block of the current through P_{2X1} purinoceptors (B). (C) The concentration-response relationship for the current inhibition. The current was measured as in (A) and (B), and the ATP-activated current at −50 mV in the presence of trivalent cations was normalized to that before the application of ATP. The data are the mean and S.E.M. obtained from 4–6 oocytes expressing P_{2X2} (circles) or P_{2X1} purinoceptors (triangles) in the presence of La^{3+} (open symbols) or Gd^{3+} (filled symbols).

4. Discussion

La^{3+} and other trivalent cations inhibited the ATP-activated current in PC12 cells and *Xenopus* oocytes expressing P_{2X2} purinoceptors at micromolar or higher concentrations. The inhibition by trivalent cations of the macroscopic ATP-activated current was in contrast to the potentiation by Zn^{2+} (Clouse et al., 1993; Li et al., 1993; Koizumi et al., 1995) or Cd^{2+} (Ikeda et al., 1996) of this current. Ca^{2+} and other divalent cations inhibited the macroscopic current, but the inhibition required millimolar concentrations of the divalent cations (Honoré et al., 1989; Nakazawa et al., 1990).

The inhibition by La^{3+} was attenuated when the concentration of ATP was increased (Fig. 2A), suggesting the reduction by La^{3+} of the apparent affinity of ATP for P_{2X} purinoceptors. The reduction in affinity cannot be attributed to simple competitive antagonism at P_{2X} purinoceptors because the slope of the concentration–response relationship became steeper in the presence of La^{3+} or Gd^{3+} (Fig. 2B,C). Some allosteric mechanism that can explain the steeper slope ('positive cooperativity') must be postulated. Another possibility that can account for the dependence of ATP is the reduction by La^{3+} of the effective concentration of ATP: La^{3+} may bind to negatively charged ATP molecules. This buffering may be effective enough for low concentrations of ATP, but it may be insufficient for higher concentrations. In fact, this mechanism was adopted as explanation for the inhibition by Ca^{2+} and other divalent cations of the ATP-activated macroscopic current in rat myometrium (Honoré et al., 1989). However, the idea of the binding of trivalent cations to ATP alone cannot completely explain the current inhibition because the current activated by 30 μM ATP was largely blocked by 1 μM Gd^{3+} in *Xenopus* oocytes expressing P_{2X2} purinoceptors (Fig. 5).

The voltage-dependent slowly activating component of the ATP-activated current was not blocked by La^{3+} (Fig. 4A,B; Table 1). The appearance of the slow component upon hyperpolarization indicates that ATP molecules bound to the receptor/channels even in the presence of La^{3+}, and, thus, may support the idea of allosteric modulation. La^{3+} may selectively block a 'voltage-independent' open

state. Another explanation for this phenomenon is that although La^{3+} inhibits the binding of ATP and reduces the voltage-dependent component as it does the voltage-independent component, this inhibitory effect is canceled by simultaneously occurring enhancement of the voltage-dependent component. Such enhancement may occur if La^{3+} screens off the surface potentials of membrane or channel proteins. The charge screening by trivalent cations at micromolar concentrations was proposed as a mechanism underlying the increase in the conductance of mechanogated non-selective cation channels (Hamill and McBride, 1996). However, the facilitation of the voltage-dependent component by this mechanism, if it exists, may not be large enough to cancel the inhibitory action of La^{3+}, because the voltage dependence of this component was not affected by La^{3+} (Fig. 4C).

Trivalent cations are known to inhibit ion permeation through various types of cation-selective channels by blocking the channel pore (Hamill and McBride, 1996; Kiss and Osipenko, 1994). As the ATP-gated channels are cation selective, this mechanism may also contribute to the current inhibition. However, pore blockade cannot be a major mechanism because (1) La^{3+} and Gd^{3+} did not significantly affect maximal current responses to ATP (Fig. 2B,C), and (2) La^{3+} failed to block the voltage-dependent component of the ATP-activated current (Fig. 4).

La^{3+} and Gd^{3+} inhibited the current through P_{2X2} purinoceptors expressed in *Xenopus* oocytes (Fig. 5). As this subclass was cloned from PC12 cells (Brake et al., 1994), the current inhibition in these cells may result from an action on the receptors of this class. La^{3+} and Gd^{3+} also inhibited the current through P_{2X1} purinoceptors expressed in *Xenopus* oocytes (Fig. 5), suggesting that the inhibition by trivalent cations is not peculiar to P_{2X2} purinoceptors. Trivalent cations may rather non-selectively inhibit subclasses of P_{2X2} purinoceptors. In this regard, the current inhibition in PC12 cells may also be attributed to other types of P_{2X} purinoceptors such as P_{2X4} purinoceptors, which have been shown to be expressed in these cells (Wang et al., 1996).

In summary, the present study has demonstrated that La^{3+} and trivalent cations inhibit the current mediated through P_{2X} purinoceptors. The current inhibition may, at least partly, be due to allosteric modulation by these cations of P_{2X} purinoceptors, although other mechanisms, such as the reduction of free concentration of ATP or the block of ion permeation at the channel pore, cannot be excluded. Most of the modulations of P_{2X} purinoceptors that have been reported are stimulatory (see Section 1); inhibitory effects have been observed with rather a small number of compounds such as competitive antagonists, high concentrations of *d*-tubocurarine (e.g., Nakazawa et al., 1991) and millimolar Ca^{2+} (Honoré et al., 1989; Nakazawa et al., 1990). The inhibition by trivalent cations occurred at concentrations as low as micromolar and, thus, this modulation may provide useful information concerning the structure of P_{2X} purinoceptors in relation to channel function, and the pharmacology of these receptors, including the development of selective antagonists. Alternatively, the inhibition may be related to the toxicology of trivalent cations, as proposed for other types of channels (Kiss and Osipenko, 1994).

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