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Cisplatin Activates Volume-Sensitive Like Chloride Channels Via Purinergic Receptor Pathways in Nasopharyngeal Carcinoma Cells

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Abstract Cisplatin-based concomitant chemoradiotherapy is considered as the standard treatment for locally advanced nasopharyngeal carcinoma patients. However, the curative efficacy of cisplatin-based chemotherapy is limited because of the occurrence of cisplatin resistance. Some researches indicate that activating the volume-sensitive Cl^- channel might be a new strategy for the reduction of cisplatin resistance. However, little is known about the activation pathway of the Cl^- channels activated by cisplatin. In this study, the cisplatin-activated chloride current was investigated using the whole cell patch-clamp technique in the poorly differentiated nasopharyngeal carcinoma cells (CNE-2Z cells), and the activation pathway of the current was also discussed. The results showed that extracellular application of cisplatin activated a Cl^- current, showing the properties of significant outward rectification, intracellular ATP dependency, and a selectivity sequence of $I^- > Br^- > Cl^- >$ gluconate, and being inhibited by the Cl^- channel inhibitors tamoxifen and extracellular ATP. These characteristics are similar to those of the volume-sensitive Cl^- current in CNE-2Z cells, indicating that cisplatin induces the Cl^- current by activating the volume-sensitive like chloride channel. The cisplatin-activated current was blocked by suramin (a wide-spectrum purinergic antagonist) and RB2 (a relatively selective P2Y antagonist). In addition, the current was depressed by extracellular application of apyrase. The apoptotic volume decrease induced by cisplatin was also attenuated by RB2. P2Y receptors were expressed in CNE-2Z cells. These results suggest that cisplatin can induce a Cl^- current by activating volume-sensitive like Cl^- channels through the P2Y purinoceptor pathway.

Keywords Patch-clamp techniques · Chloride channels · Nasopharyngeal neoplasms · Cisplatin · Purinergic receptors

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Introduction

Nasopharyngeal carcinoma is prevalent in Southeast China and Southeast Asia. Cisplatin-based concomitant chemoradiotherapy is identified as the standard treatment for locally advanced nasopharyngeal carcinoma patients (Zhou et al. 2012). However, the curative efficacy of cisplatin-based chemotherapy is restricted because of the intrinsic or acquired cisplatin resistance (Jin et al. 2012; Zhang et al. 2014). Despite a growing number of reports, the mechanism of cisplatin resistance still awaits to be elucidated clearly.

In the past few years, the relationship between the impaired activity of the volume-sensitive Cl^- channel and the cisplatin resistance has drawn increasing interest of researchers. In KCP-4 cells, which were derived from human epidermoid cancer KB cells, the volume-sensitive Cl^- currents could not be detected (Lee et al. 2007). In addition, the cisplatin-resistant A549/CDDP cells exhibited no obvious volume-sensitive Cl^- currents and apoptotic volume decrease (AVD), a major hallmark of apoptosis, when treated with cisplatin (Min et al. 2011). These findings imply that the loss of volume-sensitive Cl^- channel function is associated with the acquisition of cisplatin resistance, probably by preventing AVD and eventually apoptosis; activating the volume-sensitive Cl^- channel may be a new strategy for the reduction of clinical cisplatin resistance. However, it remains unclear about the activation pathway of the volume-sensitive Cl^- channels activated by cisplatin.

In our previous studies, we have demonstrated that volume-sensitive Cl^- channels play crucial roles in cell volume regulation, cell cycle, and cell migration in the human nasopharyngeal carcinoma cell line CNE-2Z (Chen et al. 2002, 2007; Mao et al. 2004, 2007, 2008). We have also found that cisplatin-induced AVD and apoptosis in CNE-2Z cells, which could be inhibited significantly by the Cl^- channel blocker NPPB (Fan et al. 2007), suggesting that Cl^- channels are involved in cisplatin-induced apoptosis in CNE-2Z cells by inducing AVD.

In the present study, we sought to explore the characteristics of the Cl^- current induced by cisplatin in CNE-2Z cells, and to further discuss the activation pathway of the channels activated by cisplatin. We found that cisplatin treatment activated a Cl^- current showing characteristics similar to those of the volume-sensitive Cl^- current in CNE-2Z cells. The purinergic antagonists, suramin and reactive blue 2 (RB2), suppressed the cisplatin-activated current significantly. The current was also inhibited by apyrase. These results indicate that cisplatin induced a volume-sensitive like Cl^- current through the purinergic receptors pathway.

Materials and Methods

Cell Culture

The poorly differentiated nasopharyngeal carcinoma cells (CNE-2Z) were cultured in RPMI 1640 medium with 10 % fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2, as described previously (Chen et al. 2002; Mao et al. 2004). The cells were subcultured every 2 days. For patch-clamp studies, cells were harvested after being cultured for 48 h, resuspended in culture medium, plated onto 12-mm round glass coverslips, and incubated at 37 °C for 2 h before experiments.

Patch-Clamp Experiments

The coverslips containing the prepared cells were stuck onto the base of the recording chamber (with a volume of 0.5 ml). The chamber was perfused with extracellular solutions throughout the experiment at a speed of 2 ml/min. Whole cell currents of CNE-2Z cells were recorded using a patch-clamp amplifier (EPC-7, List Electronic, Germany) at room temperature (20–24 °C), as previously described by us (Chen et al. 2002, 2007; Sun et al. 2012). Electrodes, giving a resistance of 5–10 MΩ when filled with the pipette solution, were pulled from glass capillaries with the outer diameter 1.5 mm on a two-stage vertical puller (PB-7, Narishige, Japan). The reference electrode (Ag–AgCl wire) was linked to the bath using an agar bridge. Throughout the experiments, cells were held at 0 mV (the Cl^- equilibrium potential) and then stepped to the 200-ms pulses of ±40, 0, and ±80 mV in sequence and repeatedly, with a 4-s interval between pulses. All current measurements were made 10 ms after the onset of each voltage pulse. The pulse generation and current analysis were accomplished using the EPC software package (CED, Cambridge, UK). Currents were normalized by cell membrane capacity.

The permealility ratios (P_X/P_{Cl}) of various anions (X^-) to Cl^- were calculated using the modified Goldman–Hodgkin–Katz equation, $P_X/P_{Cl} = ([Cl^-]_n \exp(-\Delta V_{rev}F/RT) - [Cl^-]_s)/[X^-]_s$, where $[Cl^-]_n$ and $[Cl^-]_s$ are the Cl^- concentration in the normal and the substituted bath solution, $[X^-]_s$ is the concentration of the substituted anion, ΔV_{rev} is the difference of the reversal potentials for Cl^- and X^-, F is the Faraday constant, R is the gas constant, and T is the absolute temperature.

The percentage of inhibition of the cisplatin-activated Cl^- current was calculated using the following equation: $\text{Inhibition}\ (\%) = [(\text{Current}_{\text{ctrl,cisplatin}} - \text{Current}_{\text{Iso}}) - (\text{Current}_{\text{blockers}} - \text{Current}_{\text{Iso}})]/(\text{Current}_{\text{ctrl,cisplatin}} - \text{Current}_{\text{Iso}}) \times 100\ \%$, where $\text{Current}_{\text{Iso}}$ is the background current under isotonic condition, $\text{Current}_{\text{ctrl,cisplatin}}$ the peak cisplatin-activated current, and $\text{Current}_{\text{blockers}}$ the current recorded after blocker treatments.

Cell Volume Measurements

Cell volume was measured at the room temperature (20–24 °C) using the method described previously (Zuo et al. 2009). Cell images were captured every 60 s by a CCD camera (CCD625, Leica, Germany). Each image was analyzed with the Scion software (Scion Corporation). The changes of cell volume caused by different treatments were

calculated using the formula $(V_{TEST} - V_{CTRL})/V_{CTRL} \times 100$ %, where V_{CTRL} stands for the basal cell volume under isotonic conditions and V_{TEST} stands for the cell volume after various treatments.

Immunofluorescence

The expression of P2Y receptor in CNE-2Z cells was detected using the technique we described before (Mao et al. 2013). Cells grown on 6 mm coverslips were fixed in 4 % paraformaldehyde for 15 min, permeabilized in 0.5 % Triton X-100 for 5 min, blocked with 10 % sheep serum, incubated in the absence and presence of the primary antibody (goat polyclonal $P2Y_2$ antibody; diluted 1:50; Santa Cruz, USA) at 4 °C overnight, followed by incubation with the secondary antibody (donkey anti-goat IgG conjugated with Cy3, diluted 1:100 in 1 % sheep serum; Beyotime Institute of Biotechnology, China) for 1 h, and observed under a Nikon Eclipse C1 confocal laser scanning microscope (Nikon Corporation, Tokyo, Japan).

Solutions and Chemicals

The pipette solution contained (in mM): 70 *N*-methyl-D-glucamine chloride (NMDGCl), 10 HEPES, 1.2 $MgCl_2$, 1 EGTA, 140 D-mannitol, and 2 ATP. The isotonic bath solution contained (in mM): 70 NaCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, and 140 D-mannitol. The osmolarity of solutions was detected by a freezing point osmometer (Osmomat 30, Gonotec, Berlin, Germany) and adjusted to 300 mOsmol/L. The pH of pipette and bath solutions was adjusted to 7.2 and 7.4, respectively, with Tris solution. 70 mM NaCl in the isotonic solution was substituted for equimolar NaI, NaBr, or sodium gluconate in anion substitution experiments.

Cisplatin was diluted to the final concentration of 5 μM with the isotonic solution. In Cl^- channel block experiments, tamoxifen was freshly dissolved in methanol and ATP in distilled water, at the concentration of 30 and 100 mM, respectively, and diluted to the final concentration of 30 μM and 10 mM, respectively, with the isotonic solution containing 5 μM of cisplatin. The purinergic receptor antagonists, suramin and reactive blue 2 (RB2), were prepared as the stock solutions of 10 and 100 mM, respectively, using distilled water, and diluted to the final concentration with the isotonic solution containing 5 μM of cisplatin. The pH of the final solutions was adjusted to 7.4.

Statistics

Data were presented as mean ± standard error. An analysis of variance (ANOVA) and *t* test were used for statistical analysis. A value of $P < 0.05$ was considered to be significant.

Results

Cisplatin-Activated Cl^- Currents in CNE-2Z Cells

Using patch-clamp techniques, the whole cell currents of CNE-2Z cells were recorded. Cells were clamped at 0 mV in the whole cell configuration and stepped to 0, ±40, and ±80 mV repeatedly. When cells were bathed in the isotonic solution, the currents were small and stable (Fig. 1a, b). However, application of cisplatin (5 μM) to the bath for 3–4 min significantly augmented the current, showing an apparent outward rectification property (Fig. 1a, c). The current did not exhibit time-dependent inactivation under the voltage steps applied. The cisplatin-activated current was reversed at the potential of -6.31 ± 0.43 mV ($n = 21$, Fig. 1d), which was close to the Cl^- equilibrium potential (−0.9 mV). In these experiments, there was no K^+ in the electrode and bath solutions, which prompts that the cisplatin-activated current is carried principally by Cl^-.

Anion Permeability of Cisplatin-Activated Cl^- Channel

When the currents were activated by extracellular application of cisplatin (5 μM) and reached the peak, the bath solution containing 70 mM Cl^- was replaced with the solution containing equimolar I^-, Br^-, or gluconate. The anion substitution shifted the reversal potential. The permeability ratios of P_I/P_{Cl}, P_{Br}/P_{Cl}, and $P_{gluconate}/P_{Cl}$, calculated from the shifts in reversal potential using the modified Goldman–Hodgkin–Katz equation, were 1.13 ± 0.04 ($P < 0.01$, $n = 6$), 1.10 ± 0.05 ($P < 0.05$, $n = 6$), 0.30 ± 0.04 ($P < 0.01$, $n = 6$), turning out the sequence of anion permeability of $I^- > Br^- > Cl^-$ > gluconate.

Intracellular ATP Dependence of Cisplatin-Activated Cl^- Current in CNE-2Z Cells

Intracellular ATP dependence of the cisplatin-activated current was examined via eliminating ATP (2 μM) from the pipette solution. When ATP was eliminated from the pipette solution, the cisplatin-activated current was decreased significantly by 72.2 ± 7.9 % ($P < 0.01$, $n = 5$). This indicates that the cisplatin-activated current is intracellular ATP dependent.

Blockage of Cisplatin-Activated Current by the Cl^- Channel Blockers Tamoxifen and ATP

Two Cl^- channel blockers, tamoxifen and ATP, were used to confirm that the cisplatin-activated current is the result of activation of Cl^- channels. The results showed that 10 mM extracellular ATP reversibly suppressed the cisplatin-activated current by 73.8 ± 6.6 % at +80 mV

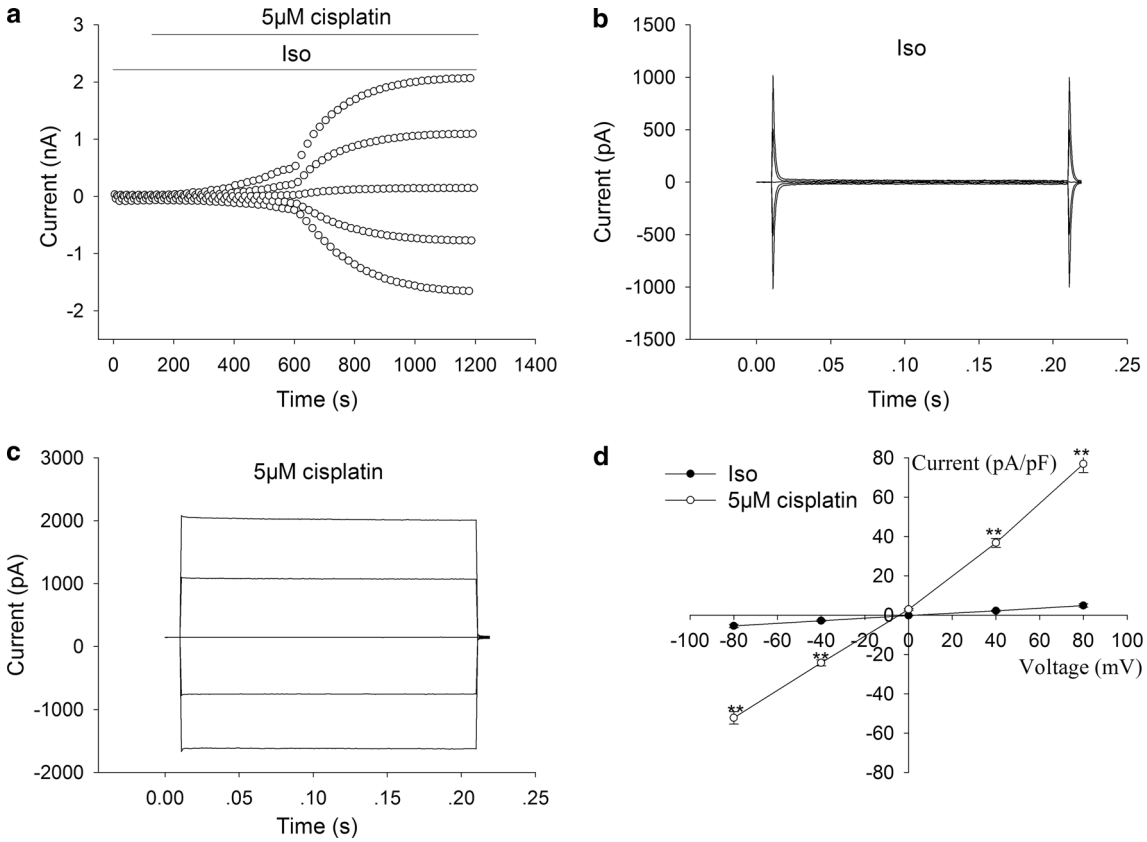

Fig. 1 Cisplatin-activated Cl^- currents in CNE-2Z cells. A typical time course of the cisplatin-activated current is shown in **a**. Typical current traces recorded under the isotonic bath solution and after extracellular application of 5 μM cisplatin are shown in **b**, **c**. Voltage was held at 0 mV and then stepped to ±40, 0, and ±80 mV. **d** The current–voltage (I–V) relationships (mean ± SE; $n = 28$). *Iso* isotonic bath solution. $^{**}P < 0.01$ (vs. current values in Iso group)

($n = 6$, $P < 0.01$) and 61.9 ± 6.4 % at −80 mV ($n = 6$, $P < 0.05$) (Fig. 2a, b). Another Cl^- channel blocker, tamoxifen (30 μM), rapidly and completely blocked the cisplatin-activated current in a reversible manner (Fig. 2c). The outward (at +80 mV) and inward (at −80 mV) currents were decreased from 57.3 ± 3.4 to 1.1 ± 0.3 pA/pF and from −28.7 ± 0.5 to −1.0 ± 0.2 pA/pF, respectively ($n = 5$, $P < 0.01$, Fig. 2d). The results indicate that the cisplatin-activated current is caused by the activation of Cl^- channels.

Blockage of Cisplatin-Activated Current by P2 Receptor Antagonists

To clarify if cisplatin induces the current by activating a P2 receptor, the effects of the P2 antagonist, suramin, on the cisplatin-activated current were first studied. Cells were exposed to cisplatin (5 μM) to activate Cl^- currents (Fig. 3a). Suramin was added to the perfusion solution when the currents had reached their peaks. Extracellular application of suramin (100 μM) inhibited the outward current at +80 mV by 93.1 ± 3.4 % ($n = 5$, $P < 0.01$) and the inward current at −80 mV by 62.7 ± 3.0 % ($P < 0.01$). The suppression of suramin on the outward currents was stronger than that on the inward currents ($P < 0.01$, Fig. 3b, c). After washing suramin out of the perfusion solution, the currents rapidly returned to their peaks. The effects of six concentrations (0.1, 1, 10, 25, 50, and 100 μM) of suramin on cisplatin-activated current were tested (Fig. 3d). Suramin inhibited the cisplatin-activated current in a dose-dependent manner, with an IC_{50} of 1.5 μM.

Suramin is a wide-spectrum purinergic antagonist, while RB2 is a relatively selective P2Y antagonist. To further confirm the subtype of the purinergic receptor, RB2 was used. Extracellular application of RB2 (100 μM) also reversibly depressed both the outward and inward currents activated by cisplatin; 61.86 ± 4.3 % of the outward current at +80 mV and 54.7 ± 4.5 % of the inward current at −80 mV were blocked ($n = 7$, $P < 0.01$, Fig. 4a, b). The inhibition of outward currents shows no significant difference from that of inward currents ($P > 0.05$). The inhibitory effects of RB2 on cisplatin-activated currents displayed dose dependence when RB2 concentration was

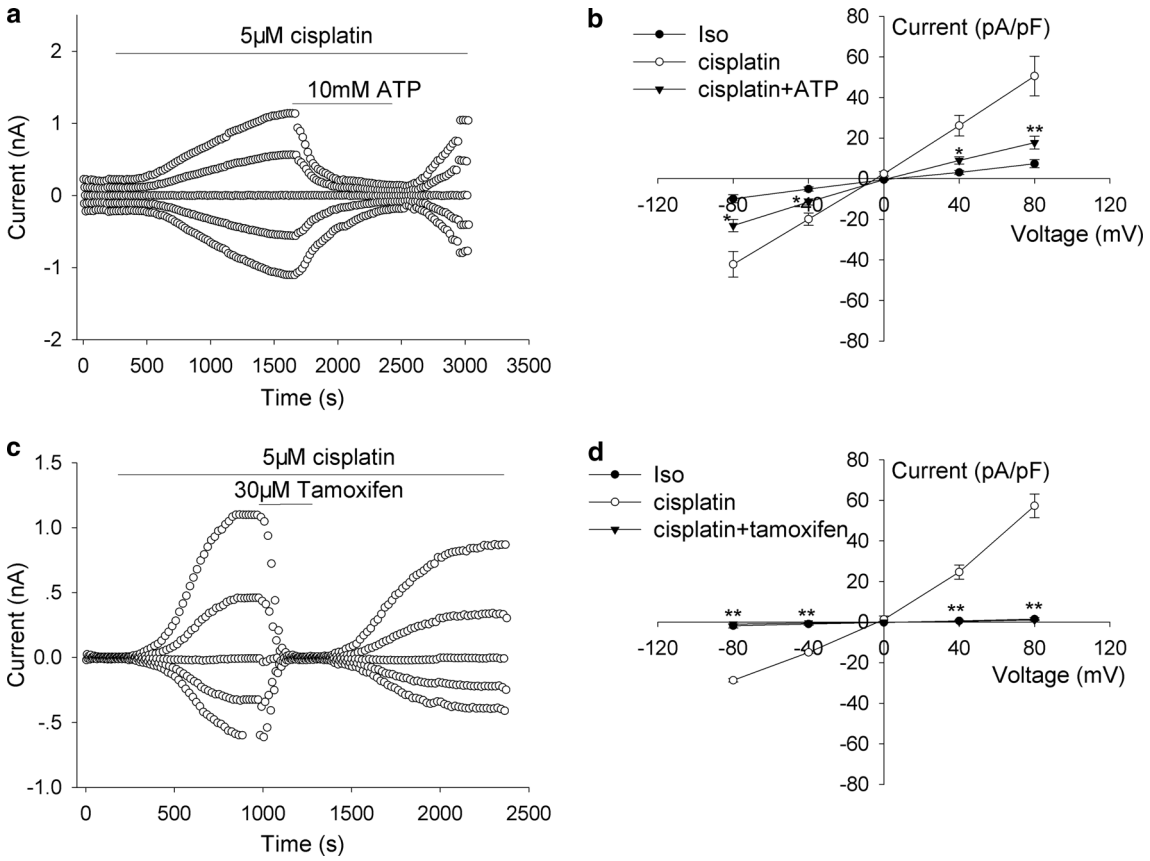

Fig. 2 Effects of the chloride channel blockers ATP and tamoxifen on cisplatin-activated Cl^- currents. The time course of the activation of the cisplatin-activated Cl^- currents and the effects of extracellular application of 10 mM ATP or 30 μM tamoxifen on the currents are shown in **a**, **c**. The current–voltage (*I–V*) relationships are presented in **b**, **d** (mean ± SE; $n = 5$ or 6). $^*P < 0.05$, $^{**}P < 0.01$ (vs. cisplatin)

increased from 0.1 to 100 μM (Fig. 4a, c), with an IC_{50} of 10.0 μM. The data suggest that cisplatin activates the current probably via a P2Y receptor pathway.

Effects of Apyrase on Cisplatin-Activated Currents

To investigate whether the cisplatin-activated current is mediated by ATP release, the actions of apyrase were examined. When apyrase (20 U/ml) was pre-added to the perfusion solution for 20 min, the current induced by cisplatin was smaller than that under the control condition (without pre-addition of apyrase) ($n = 3$, $P < 0.05$, Fig. 5a, b). The cisplatin-activated current was inhibited by 52.0 ± 4.6 % at +80 mV and 50.5 ± 9.4 % at −80 mV, respectively, with no significant difference between them ($P > 0.05$, Fig. 5c). After washing apyrase out of the bath, the current was increased in the same cells (data not shown). These data serve as evidence that cisplatin can activate Cl^- channels through stimulating the release of ATP, which then activates the P2 receptors.

Suppression of Cisplatin-Induced Apoptotic Volume Decrease by the P2Y Antagonist RB2

Apoptotic volume decrease (AVD) is a major hallmark of cell apoptosis, upstream to the biochemical apoptotic events (such as cytochrome C release, caspase-3 activation, and DNA laddering). To further investigate the role of P2 receptors in AVD, the effects of the P2Y antagonist, RB2 on cisplatin-induced AVD were studied. As illustrated in Fig. 6a, cell volume was stable under the isotonic condition (control, $n = 12$). When treated with cisplatin (5 μM), the cells were gradually shrunk. Cell volume was decreased to 93.4 ± 0.4 and 87.0 ± 0.5 % at 60 and 120 min, respectively ($n = 41$, Fig. 6b). Conversely, when cells were challenged with the mixture of RB2 (100 μM) and cisplatin (5 μM) for 120 min, no apparent AVD was observed (Fig. 6a, b). The cell volume was not significantly different from that in the control group ($P > 0.05$, Fig. 6b). These results demonstrate that apoptotic volume decrease induced by cisplatin is prevented by RB2, a relatively selective P2Y antagonist, suggesting that the P2Y signal pathway is

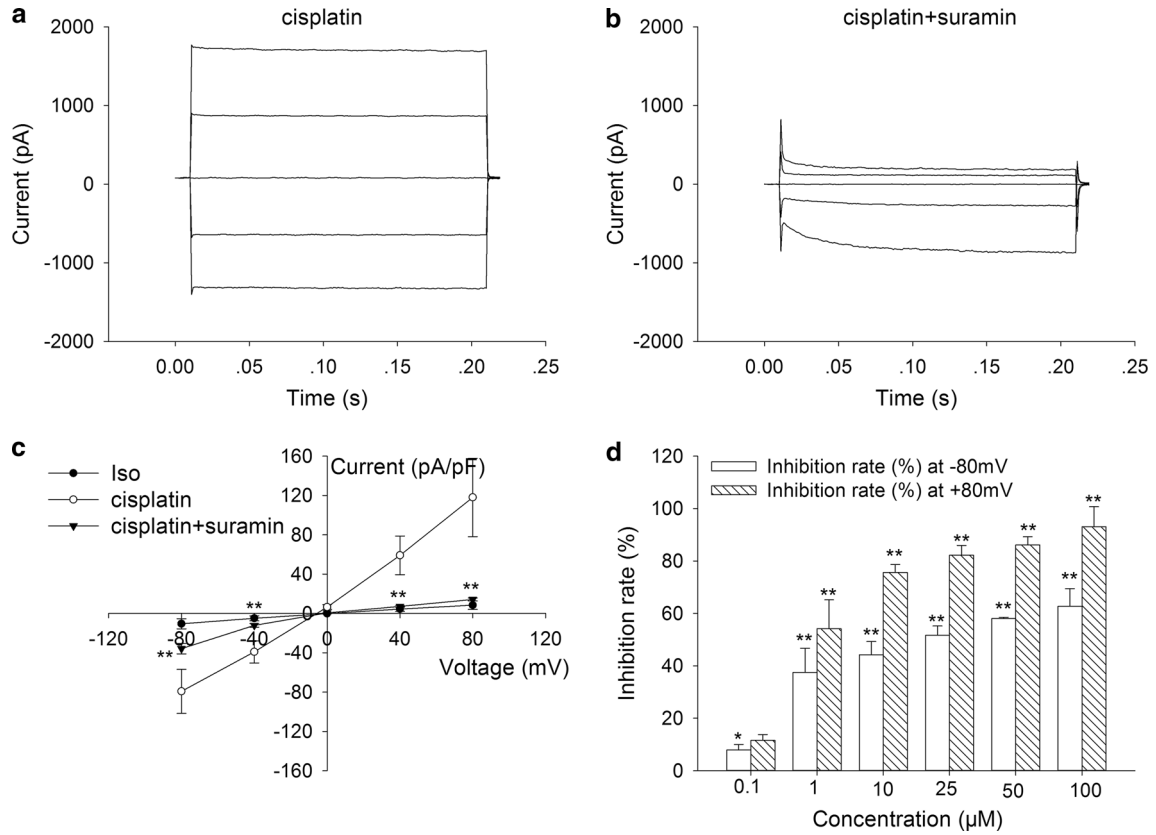

Fig. 3 Inhibition of cisplatin-activated currents by the P2 purinergic receptor antagonist suramin. Extracellular application of cisplatin (5 μM) activated a current (**a**), which was inhibited by suramin (100 μM, **b**). **c** The *I–V* relationship (mean ± SE; $n = 5$). **d** Inhibition rates of cisplatin-activated chloride currents by different doses of suramin at +80 and −80 mV (mean ± SE; $n = 4$ or 5). $^{*}P < 0.05$, $^{**}P < 0.01$ (vs. cisplatin)

functionally involved in the cisplatin-induced AVD and apoptosis.

P2Y Receptor Expression in CNE-2Z Cells

The expression of P2Y receptors in CNE-2Z cells was detected by immunofluorescence. The results showed that P2Y receptors were expressed in CNE-2Z cells (Fig. 7). P2Y receptors were distributed on the cell membrane as well as in the cells.

Discussion

In the present study, we first described a Cl^- current activated by extracellular application of cisplatin in CNE-2Z cells. The current showed the properties of significant outward rectification, intracellular ATP dependency, a selectivity sequence $I^- > Br^- > Cl^- >$ gluconate, and being blocked by the Cl^- channel inhibitors tamoxifen and extracellular ATP. Many of the characteristics of this cisplatin-activated Cl^- current are similar to those of the volume-sensitive Cl^- current that we have reported before (Chen et al. 2002, 2007; Mao et al. 2004), suggesting that the channel activated by cisplatin might be the same type as the volume-sensitive Cl^- channel. The cisplatin-activated current was abolished by the purinergic antagonists, suramin and RB2, and was suppressed by apyrase. These results indicate that purinergic receptors might mediate the activation of Cl^- channels induced by cisplatin in CNE-2Z cells.

Volume-sensitive Cl^- channels are considered to play an essential role in the mechanism of cisplatin-induced apoptosis due to the facts that cisplatin induced a Cl^- current with properties similar to the volume-sensitive Cl^- current in human epidermoid cancer KB cells (Ise et al. 2005; Shimizu et al. 2008) and wild-type A549 cells (Min et al. 2011). In the present study, cisplatin also induced a Cl^- current sharing the similar properties with the volume-sensitive Cl^- current in CNE-2Z cells. This finding, along with our previous finding that Cl^- channels participated in the cisplatin-induced AVD and apoptosis in CNE-2Z cells (Fan et al. 2007), suggests that cisplatin might activate volume-sensitive Cl^- channels, leading to AVD and eventually apoptosis in CNE-2Z cells.

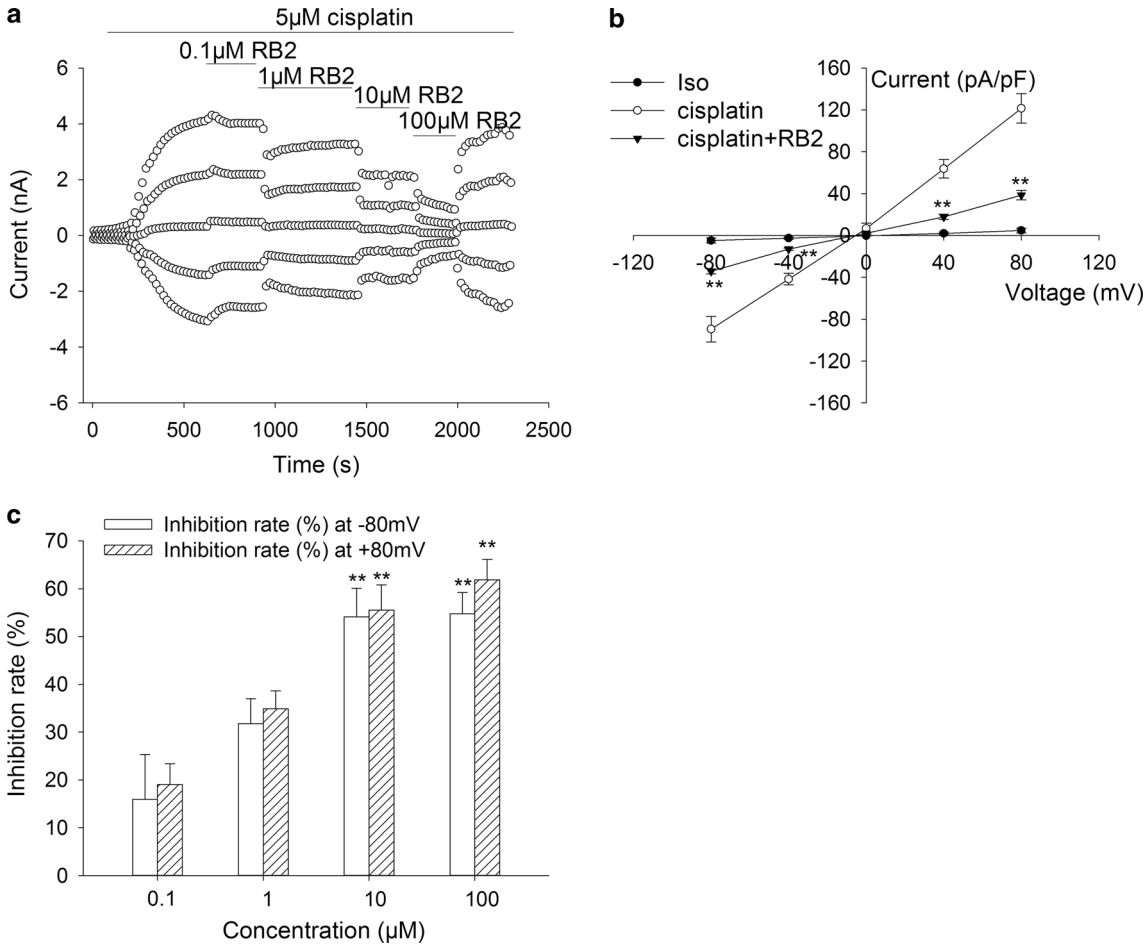

Fig. 4 Inhibition of cisplatin-activated currents by the P2Y purinergic receptor antagonist RB2. The time course of activation of the cisplatin-activated Cl^- currents and the effects of extracellular application of different concentrations of RB2 (0.1, 1, 10, and 100 μM) on the currents are shown in **a**. The *I–V* relationships are presented in **b** (mean ± SE; $n = 5$). **c** Inhibition rates of cisplatin-activated chloride currents by different doses of RB2 at +80 and −80 mV (mean ± SE; $n = 7$). $^{**}P < 0.01$ (vs. cisplatin)

Cisplatin is a platinum-based anticancer agent that is widely used in chemotherapy for various malignancies (Kasai et al. 2013). However, the occurrence of the cisplatin resistance restricts its curative efficacy in cancer chemotherapy. The mechanism of cisplatin resistance is extremely complicated, including defective endocytic uptake, changes in chromatin structure, aberrant DNA-methylation, and histone acetylation, mutation induced by many transcription factors and microRNAs, gene activation/silencing, activation of the epithelial to mesenchymal transition pathway, inactivation of the apoptosis pathways, and increased/decreased expression of membrane transporters, heat shock proteins, small GTPase and ribosomal proteins, exosomes, and others (Almeida et al. 2014; Shen et al. 2012; Tan et al. 2014; Xiao et al. 2014; Zhang et al. 2014). Recently, the loss of volume-sensitive Cl^- channel function was found to be related to the acquisition of cisplatin resistance via preventing AVD and eventually apoptosis (Lee et al. 2007; Min et al. 2011), which gives us a hint that studies on the activation pathway of Cl^- channels activated by cisplatin will make contributions to cancer chemotherapy.

In the present study, we found that the cisplatin-activated current can be abolished by suramin, a wide-spectrum purinergic antagonist, and RB2, a relatively selective P2Y antagonist, suggesting that the activation of Cl^- channels by cisplatin might be mediated by P2Y receptors in CNE-2Z cells. The suppression of cisplatin-induced AVD by RB2 gives evidence from another perspective. Although ATP released from CNE-2Z cells was not observed directly, the actions of apyrase were studied in this study. Our results demonstrated that the cisplatin-activated Cl^- current was depressed by the pre-addition of apyrase. These data suggest that the induction of the Cl^- current by cisplatin might be the result of ATP release, which activates the Cl^- channels via stimulating P2Y receptors.

Suramin is a nonselective P2 receptor antagonist against P2X and P2Y, while RB2 is a specific P2Y antagonist

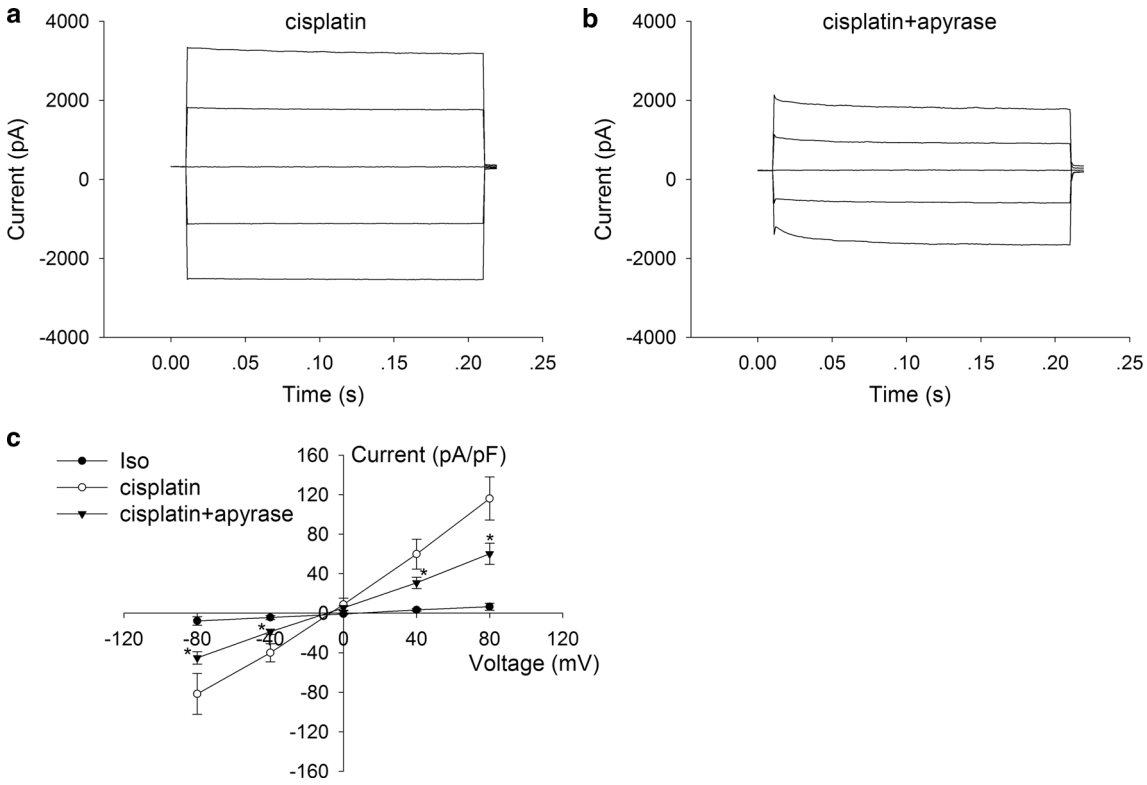

Fig. 5 Inhibition of cisplatin-activated currents by apyrase. Extracellular application of cisplatin (5 μM) activated a current (**a**), which was inhibited by apyrase (20 U/ml, **b**). The *I–V* relationships are presented in **c** (mean ± SE; $n = 5$). *$P < 0.05$ (vs. cisplatin)

(Darby et al. 2003). In the present study, our results show that the blockage effects of suramin on the cisplatin-activated current were greater than those of RB2, implying that in addition to P2Y receptors, P2X receptors are involved in this process. There are seven subforms of P2X receptors ($P2X_{1–7}$) that have been identified (Mehta et al. 2014). It is well known that $P2X_7$ receptor plays an important role in inducing apoptosis (Mehta et al. 2014). As the close relationship between the volume-sensitive chloride channel and apoptosis, the role of P2X receptors in the activation of chloride channel has aroused researchers' interest. In astrocytes and lymphocytes, P2X receptors are not involved in activating volume-sensitive Cl^- channels (Darby et al. 2003; Ma et al. 2004). Opposite to the results in these two cells, $P2X_4$ plays a modulatory role in volume-sensitive Cl^- current activation in rat hepatoma cells (Varela et al. 2010). In this work, we found that the cisplatin-activated current was very sensitive to the putative P2 receptor antagonist suramin ($IC_{50} = 1.5$ μM). According to the IC_{50} of suramin on the P2X receptor subtypes $P2X_{4–7}$ (Boarder and Hourani 1998), $P2X_{4–7}$ might not be involved in the activation of the cisplatin-activated current, but the involvement of other subtypes of P2X receptors cannot be excluded. It remains to be further explored if other P2X receptor subtypes are functional in the activation of the cisplatin-activated current. In this study, it was found that P2Y receptors were expressed in the CNE-2Z cells. So far, a total of eight P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) have been cloned and characterized (Moheimani and Jackson 2012). The antibody used in our experiments is more selective for $P2Y_2$. It is possible that $P2Y_2$ is an important element in the activation pathway of the cisplatin-activated current although this remains to be clarified. Because of the insensitivity of $P2Y_4$ receptors to suramin (Boarder and Hourani 1998), the possible involvement of this subtype could be eliminated. Whether other subtypes of P2Y receptors are involved in the activation of the cisplatin-activated current remains to be further studied.

In the studies of the activation of Cl^- channels during cellular swelling, Darby et al. (2003) demonstrated that the stimulation of P2 receptors contributes to the activation of swelling activated Cl^- currents in astrocytes because the current was blocked by suramin and RB2, and depressed by apyrase. The similar results could also be found in HTC rat hepatoma cells (Roman et al. 1997; Wang et al. 1996), cultured human and rat biliary epithelial cells (Roman et al. 1999) and human hepatocytes (Feranchak et al. 2000). However, Okada et al. disagreed with the hypothesis that stimulation of P2 receptors by released ATP leads to the

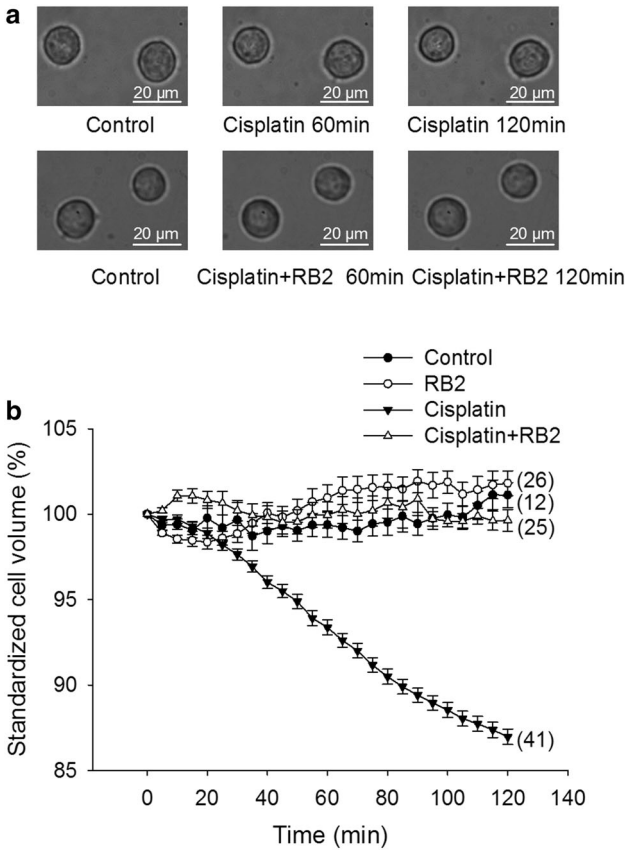

Fig. 6 Inhibition of cisplatin-induced apoptotic volume decrease by the P2Y purinergic receptor antagonist RB2. Cells were incubated in the isotonic control solution (control) or in the isotonic solution containing 100 μM RB2 (RB2), 5 μM cisplatin (cisplatin), or 5 μM cisplatin plus 100 μM RB2 (cisplatin + RB2) for the indicated time periods. The typical cell images are shown in **a**, and the changes of standardized cell volume under different conditions are presented in **b**

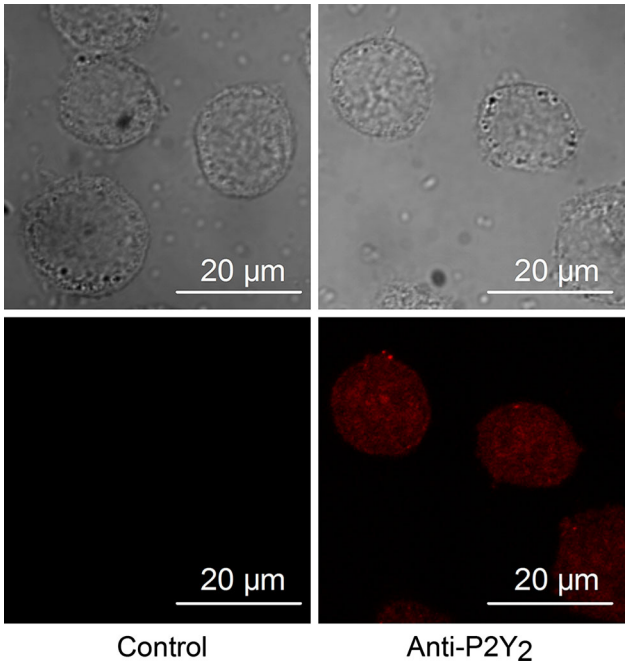

Fig. 7 Expression of P2Y receptors detected by immunofluorescence in CNE-2Z cells. The *top* two images are transmitted light images. The *bottom* images are confocal fluorescent images of the control cells and the cells treated with the anti-$P2Y_2$ antibody

activation of volume-sensitive Cl^- channels, based on the following three lines of evidence: first, extracellular application of ATP could not activate volume-sensitive Cl^- channels in some types of cells, such as human intestine 407 cells; second, C127/CFTR cells exhibited significant background ATP-release activity but never showed sizable Cl^- currents under isotonic conditions; and third, Gd^{3+} or anti-ATP-release antibodies, which could block swelling-induced ATP release, could not inhibit swelling-induced activation of volume-sensitive Cl^- channels in intestine 407 cells (Okada et al. 2001). However, our previous studies have shown that extracellular application of ATP activated a current with the properties similar to those volume-activated chloride current in CNE-2Z cells and the current was depressed by RB2, suggesting that the chloride channel can be activated by extracellular ATP through purinoceptor P2Y (He et al. 2004). In addition, a background Cl^- current was recorded under the isotonic condition in CNE-2Z cells; the current could be activated by paracrine ATP through purinergic receptor pathways, and was involved in cell volume regulation (Yang et al. 2011). Furthermore, the cisplatin-activated Cl^- current was depressed partially by apyrase in this study. We thus put forward a hypothesis that cisplatin activates volume-sensitive like chloride channels via this pathway: the cisplatin treatment causes the release of ATP from CNE-2Z cells, which then binds to the P2Y receptor, leading to activation of chloride channels.

In summary, we found in the present study that cisplatin induces a chloride current sharing the properties similar to those of the volume-activated chloride current in CNE-2Z cells. The activation of the cisplatin-activated chloride current is dependent on the stimulation of P2Y purinoceptors by ATP released from cells. These findings help to elucidate the action mechanism of cisplatin from a new perspective, which will result in the advance of more effective treatments for cancer.

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