Effects of Antiarrhythmic Drugs on the Hyperpolarization-Activated Cyclic Nucleotide–Gated Channel Current

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Abstract. After the report of the Cardiac Arrhythmia Suppression Trial, a tabular framework of the Sicilian Gambit has been proposed to display actions of antiarrhythmic drugs on ion channels and receptors and to provide more rational pharmacotherapy of arrhythmias. However, because effects of antiarrhythmic drugs on \(I_f\) have not been thoroughly examined, we used patch clamp techniques to determine the effects of various antiarrhythmic drugs on the HCN (hyperpolarization-activated cyclic nucleotide–gated) channel currents. HCN4 channels, a dominant isoform of HCN channels in the heart, were expressed in HEK293 cells. Amiodarone and bepridil potently inhibited the HCN4 channel current with \(IC_{50}\) values of 4.5 and 4.9 \(\mu M\), respectively, which were close to their therapeutic concentrations. The inhibitory effects of quinidine, disopyramide, cibenzoline, lidocaine, mexiletine, aprindine, propafenone, flecainide, propranolol, and verapamil on the HCN4 channel current were weak in their therapeutic concentrations, with \(IC_{50}\) values of 78.3, 249, 46.8, 276, 309, 43.7, 14.3, 1700, 50.5, and 44.9 \(\mu M\), respectively, suggesting that the inhibitory effects on \(I_f\) would be clinically small. d,l-Sotalol hardly affected the HCN4 channel current. Information about the HCN4-channel effects of many antiarrhythmic drugs may be useful for determining the appropriate drug for treatment of various arrhythmias while minimizing adverse effects.

Keywords: antiarrhythmic drug, hyperpolarization-activated cyclic nucleotide–gated (HCN) channel subtype 4, pacemaker current, amiodarone, bepridil

Introduction

Pacemaker current was functionally identified in sino-atrial (SA) node cells about three decades ago (1, 2). This current, named \(I_f\) or \(I_h\), is a mixed Na\(^+\) and K\(^+\) inward current (3), which flows through the hyperpolarization-activated cyclic nucleotide–gated (HCN) channels. This channel has atypical features: unlike most voltage-gated channels, the HCN channel opens upon membrane hyperpolarization with unusually slow kinetics. Recently molecular cloning has identified four subtypes of HCN channels (HCN 1 – 4) in mammals (4 – 7). Three isoforms have been identified in cardiac tissues, HCN 1, 2 and 4, with HCN4 being the dominant one (4, 7, 8). In addition to SA node cells, \(I_f\) has been considered to produce automatic activity from other cardiac regions such as Purkinje fibers, atrioventricular (AV) node, atrium, and ventricle (9 – 11). The latent pacemakers arising from phase 4 depolarization (slow diastolic depolarization) play a compensatory role in pacemaking when SA or AV node function is impaired. However, excessive activation of \(I_f\) in cardiac regions other than the SA node may elicit abnormal automaticity from the ectopic focus, resulting in atrial and ventricular arrhythmias (12 – 15). It was demonstrated that the \(I_f\) densities in left ventricular myocytes were increased in hypertrophied hearts or end-stage failing hearts, leading to an increased propensity of ventricular arrhythmias (12 – 14). Indeed, in an experimental canine model of heart failure, HCN4 expression but not HCN2 expres-
sion in the right atrium was significantly upregulated at mRNA and protein levels, although both HCN4 and HCN2 expression in the SA node were downregulated (16). In addition, Stilitilano et al. (17) reported that both mRNA and protein levels of HCN2 and HCN4 channels were increased several fold in the atrium and the ventricle of failing human hearts. In the study, HCN4 mRNA was more strongly expressed than HCN2 mRNA; and the electrophysiological properties of $I_f$ recorded from failing ventricular myocytes, resembled those of HCN4 channels (17). Therefore, it is important to examine the effects of drugs on HCN4 channels, although the assembly of HCN isoforms in native $I_f$ channels has not been established.

The Vaughan Williams classification of antiarrhythmic drugs (18) has been used widely by clinicians, cardiologists, and researchers for a long time. After the report of the Cardiac Arrhythmia Suppression Trial (CAST) (19), a two-dimensional tabular framework of the Sicilian Gambit has been proposed to display actions of antiarrhythmic drugs on ion channels and receptors (20). However, effects of antiarrhythmic drugs on $I_f$ have not been thoroughly examined, and only alinidine and aprindine were shown to inhibit the current (20, 21). Information about the effects of antiarrhythmic drugs on the pacemaker current would be useful for a more rational use of antiarrhythmic drugs in the clinical setting. The purpose of this study was to examine the effect of various antiarrhythmic drugs on the HCN4 channel current using patch clamp techniques. By doing so, we hoped to provide some important insights into the electrophysiological effects of antiarrhythmic drugs.

**Materials and Methods**

**Expression of HCN4 channels in HEK293 cells**

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 100 U/ml penicillin G, 100 mg/ml streptomycin, and 600 μg/ml zeocin (all three antibiotics from Invitrogen) and maintained at 37°C in a humidified atmosphere with 95% air and 5% CO$_2$. Full-length cDNA of rabbit HCN4 (7) was ligated to the mammalian expression vector pcDNA-3.1/Zeo (Invitrogen). HEK293 cells were transfected with this plasmid using Lipofect AMINE PLUS (Invitrogen) followed by selection and propagation in the Dulbecco’s modified Eagle’s medium. The cultures were passed every 3–5 days by use of a brief trypsin treatment. The cells were maintained at 37°C in 5% CO$_2$ and plated on collagen-coated glass cover slips 2–3 days before the electrophysiological experiments.

**Electrophysiology**

Whole-cell membrane current recordings were performed by the patch-clamp method, as described previously (22, 23). HEK293 cells were placed in a recording chamber (1-ml volume) attached to an inverted microscope (IMT-2; Olympus, Tokyo), and superfused with the HEPES-Tyrode solution at a rate of 3 ml/min. The temperature of the external solution was kept constant at 36 ± 1°C. Glass patch pipettes with a tip diameter of 2–3 μm were heat-polished and filled with an internal solution composed of 110 mM KOH, 110 mM L-aspartate, 20 mM KCl, 1 mM MgCl$_2$, 5 mM ATP-K$_2$, 5 mM phosphocreatine-K$_2$, 10 mM EGTA, and 5 mM HEPES-KOH (pH 7.4). The free Ca$^{2+}$ concentration in the pipette solution was adjusted to pCa 8. In the experiments to examine effects of antiarrhythmic drugs on HCN4 channel current, cAMP (0.3 mM) was added to the pipette solution. The resistance of the pipette filled with the internal solution was 4–8 MΩ. After the gigaohm seal between the tip and the cell membrane was formed, the membrane patch was broken by applying more negative pressure to make the whole-cell voltage-clamp mode. The electrode was connected to a patch/whole-cell clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo). Recording signals were filtered at 1-kHz band width, and series resistance was compensated by 40% – 70%. Voltage command pulses were generated, and data were acquired by a personal computer using pCLAMP software (Axon Instruments, Foster City, CA, USA). Current signals were digitized with a sampling interval of 2 kHz and stored on the hard disc of the computer. A liquid junction potential between the internal solution and the bath solution of ~8 mV was corrected. Effects of various drugs on the HCN4 channel current were evaluated approximately at 5 min after application.

**Drugs**

Drugs used in this study and their solvents were as follows: zatebradine hydrochloride (Boehringer Ingelheim Japan, Tokyo), aprindine (Bayer Schering Pharma, Osaka), cibenzoline (Astellas Pharmaceutical Co, Tokyo), mexiletine hydrochloride (Boehringer Ingelheim Japan), propafenone hydrochloride (Astellas), $d,l$-propranolol hydrochloride (Wako Pure Chemical Industries, Osaka), quinidine (Sigma Chemical), $d,l$-sotalol hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), and verapamil hydrochloride (Wako) were each dissolved in distilled water. Disopyramide (Wako), bepridil hydrochloride (Daiichi-Sankyo, Tokyo), flecainide (Eisai Pharma-
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ceutical Co., Tokyo), and lidocaine (Wako) were each dissolved in dimethyl sulphoxide (DMSO); the final concentration of DMSO was less than 0.1% during the experiments. Amiodarone (Taisho Pharmaceutical, Tokyo) was dissolved in absolute ethanol at a concentration of 10 mM and then added to the bath solution containing bovine serum albumin (0.03% – 1%), as previously described (24). The final concentration of DMSO was less than 0.1% during the entire experiment.

Statistics

All data are presented as the mean ± S.E.M. Student’s t-test was used for statistical analysis of the paired observations, and an analysis of variance (ANOVA) was performed to test the difference among the groups; A P value <0.05 was considered statistically significant. The concentration–effect data were fitted and IC$_{50}$ values were obtained using Delta Graph Professional (Delta Point, Polaroid Computing, Tokyo).

Results

HCN4 channel currents recorded from HEK 293 cells

Membrane currents were recorded from HEK 293 cells expressing HCN4 channels. Membrane currents were elicited by hyperpolarizing pulses of 2000 ms from a holding potential of −20 mV to voltages from −30 to −140 mV at 0.1 Hz and then clamp back to 0 mV for 800 ms. When cAMP (0.3 mM) was included in the pipette solution, the activation curve was shifted toward positive voltages (Fig. 1). The membrane potential of half-maximal activation (V$_{1/2}$) for the HCN4 channel current was −90.1 ± 0.6 mV and −65.4 ± 1.6 mV in the absence and presence of cAMP, respectively. Inclusion of cAMP in the pipette solution produced the hyperpolarization-induced current at physiological voltage ranges. Thereafter, we examined effects of various drugs on the HCN4 channel current using the cAMP-containing pipette solution. The HCN4 channel current was readily blocked by 3 mM Cs$^+$, as shown in Fig. 2. Zatebradine, a bradycardiac agent (25), potently inhibited the HCN4 channel current in HEK293 cells, with an IC$_{50}$ value 1.1 μM. These electrophysiological properties are consistent with those described in a previous report (7).

Effects of antiarrhythmic drugs on HCN4 channel currents in HEK293 cells

Effects of class Ia antiarrhythmic drugs, quinidine, disopyramide, and cibenzoline, on the HCN4 channel current were examined in HEK293 cells. Quinidine produced a modest reduction of the HCN4 channel current at a concentration of 30 μM (Fig. 3). The calculated IC$_{50}$ value of quinidine for inhibiting the HCN4 channel current was 78.3 μM, which was higher than the therapeutic concentration of quinidine (Table 1). Cibenzoline and disopyramide also inhibited the HCN4 channel current weakly, with calculated IC$_{50}$ values of 46.8 and 249 μM, respectively (Fig. 3), which were both higher than the therapeutic concentrations (Table 1).

Effects of class Ib antiarrhythmic drugs, lidocaine,
Fig. 2. Inhibitory effects of Cs⁺ and zatebradine on HCN4 channel current in HEK293 cells. Representative current traces before and after Cs⁺ (3 mM) or zatebradine (10 μM) are indicated in the left and middle panels, respectively. The voltage protocol is indicated in the inset. Current–voltage relationships for the fully activated channel currents measured at the end of 2000-ms hyperpolarizing pulses before and after Cs⁺ or zatebradine are indicated in the right panels. Each reported value is the mean ± S.E.M. of 7 cells for Cs⁺ experiments and 6 cells for zatebradine experiments. *P<0.05 vs. control value.

Fig. 3. Inhibitory effects of class Ia drugs, quinidine, disopyramide, and cibenzoline, on HCN4 channel current in HEK293 cells. Representative current traces before and after quinidine (30 μM), disopyramide (300 μM), or cibenzoline (30 μM) are indicated on the left side. The voltage protocol is indicated in the inset. Current–voltage relationships for the fully activated channel currents measured at the end of 2000-ms hyperpolarizing pulses before and after class Ia drugs are indicated in the right panels. Concentration–response curves for the inhibitory effects of quinidine, disopyramide, and cibenzoline on HCN4 channel current are also shown on the right. Percent inhibition of the current after a hyperpolarizing pulse to −70 mV was used as an index of block, and the IC₅₀ values were calculated. Values are each the mean ± S.E.M. of 5–6 experiments. *P<0.05 vs. control value.
mexiletine, and aprindine, on the HCN4 channel current were also examined in HEK293 cells. Lidocaine at a concentration of 30 μM inhibited the HCN4 channel current, especially at hyperpolarizing voltages below −100 mV (Fig. 4). The inhibitory effect of lidocaine on the current at −70 mV was minimal and the calculated

Table 1. IC_{50} values for antiarrhythmic drugs in inhibiting HCN4-channel current and their therapeutic concentrations

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<th>Ib</th>
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<tr>
<td>Drug</td>
<td>Quinidine 78.3 μM</td>
<td>Disopyramide 249 μM</td>
<td>Cibenzoline 46.8 μM</td>
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<td></td>
<td>Lidocaine 276 μM</td>
<td>Mexiletine 309 μM</td>
<td>Aprindine 43.7 μM</td>
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<td></td>
<td>Propafenone 14.3</td>
<td>Flecaïnine 1700 μM</td>
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<td></td>
<td>Therapeutic</td>
<td>6.1 – 15.4 μM</td>
<td>5.9 – 14.7 μM</td>
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<td>concentration</td>
<td>0.66 – 0.92 μM</td>
<td>6.4 – 21.3 μM</td>
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Therapeutic concentrations were calculated from the therapeutic plasma concentrations reported in the literature (21, 27, 28).

Fig. 4. Inhibitory effects of class Ib drugs, lidocaine, mexiletine, and aprindine, on HCN4 channel current in HEK293 cells. Representative current traces before and after lidocaine (30 μM), mexiletine (300 μM), or aprindine (10 μM) are indicated on the left side. The voltage protocol is indicated in the inset. Current–voltage relationships for the fully activated channel currents measured at the end of 2000-ms hyperpolarizing pulses before and after class Ib drugs are indicated in the right panels. Concentration–response curves for the inhibitory effects of lidocaine, mexiletine, and aprindine on HCN4 channel current are also shown on the right. Percent inhibition of the current after a hyperpolarizing pulse to −70 mV was used as an index of block, and the IC_{50} values were calculated. Values are each the mean ± S.E.M. of 5 – 7 experiments. *P<0.05 vs. control value.
IC\textsubscript{50} value was 276 \(\mu\text{M}\). Mexiletine and aprindine also weakly inhibited the HCN4 channel current (Fig. 4). The calculated IC\textsubscript{50} values of mexiletine and aprindine for inhibiting the HCN4 channel current were 309 and 43.7 \(\mu\text{M}\), respectively. The IC\textsubscript{50} values of class Ib drugs on HCN4 channels were higher compared to the therapeutic concentrations (Table 1).

We also examined effects of class Ic antiarrhythmic drugs, propafenone and flecainide, on HCN4 channel current in HEK293 cells. Propafenone at a concentration of 30 \(\mu\text{M}\) moderately inhibited the HCN4 channel current, with a calculated IC\textsubscript{50} value of 14.3 \(\mu\text{M}\), which was relatively close to the therapeutic concentration of propafenone (Table 1 and Fig. 5). However, the inhibitory effect of flecainide on the HCN4 channel current was very weak and the calculated IC\textsubscript{50} value was 1700 \(\mu\text{M}\), which was much higher than the therapeutic concentration (Fig. 5 and Table 1).

Effect of the class II antiarrhythmic drug propranolol on the HCN4 channel current was examined. The calculated IC\textsubscript{50} value of propranolol for inhibiting the HCN4 channel current was 50.5 \(\mu\text{M}\), which was also much higher than the therapeutic concentration (Fig. 5 and Table 1).

Effect of class III antiarrhythmic drugs, amiodarone and \(d,l\)-sotalol, on the HCN4 channel current was examined in HEK293 cells. Amiodarone potently inhibited the HCN4 channel current (Fig. 6). The IC\textsubscript{50} value of amiodarone for inhibiting the HCN4 channel current at \(-70\) mV was 4.5 \(\mu\text{M}\), which was very close to the therapeutic concentration (Table 1). In contrast, \(d,l\)-sotalol at 1 – 300 \(\mu\text{M}\) hardly inhibited the HCN4 channel current at \(-70\) mV, although the drug slightly inhibited the current at hyperpolarizing voltages below \(-100\) mV (Fig. 6). Therefore, the IC\textsubscript{50} value of \(d,l\)-sotalol for inhibiting the HCN4 channel current at \(-70\) mV
We examined effects of the class IV antiarrhythmic drugs, verapamil and bepridil, on the HCN4 channel current in HEK293 cells. Bepridil effectively inhibited the HCN4 channel current at voltages between $-60$ and $-120$ mV (Fig. 7). The calculated IC$_{50}$ value of bepridil for inhibiting the HCN4 channel current at $-70$ mV was $4.9 \mu M$, which was close to the therapeutic concentration (Table 1). Verapamil weakly inhibited the HCN4 channel current, especially at hyperpolarizing voltages below $-100$ mV (Fig. 7). The calculated IC$_{50}$ value of verapamil for inhibiting the HCN4 channel current at $-70$ mV was $44.9 \mu M$, which was much higher than the therapeutic concentration (Table 1).

### Discussion

The HCN4 channels expressed in HEK293 cells showed electrophysiological properties that were consistent with those reported previously (7, 26). The hyperpolarizing voltage steps activated slowly, developing inward currents that were sensitive to Cs$^+$, and the activation curve was shifted toward the positive direction by intracellular loading of cAMP. To our knowledge, however, effects of antiarrhythmic drugs on HCN channels have not been examined. In this study we have examined for the first time the effects of various antiarrhythmic drugs on the HCN4 channel current.

In the present study we used the cAMP-containing pipette solution because we expected the tonic stimulation of the sympathetic nerve system would be observed in the heart in situ and the cardiac cells would contain some amount of cAMP in the cytosol. Inclusion of cAMP in the pipette solution produced the hyperpolarization-induced current at physiological voltage ranges around $-70$ mV, and the IC$_{50}$ values were calculated from the inhibitory effects of antiarrhythmic drugs on the HCN4 channel current evoked by the hyperpolarization pulse to $-70$ mV.

In this study, amiodarone and bepridil potently inhibited the current through HCN4 channels expressed in HEK293 cells with IC$_{50}$ values of 4.5 and 4.9 $\mu M$, respectively. Since these IC$_{50}$ values were close to their respective therapeutic concentrations (25, 26), the inhibition of $I_f$ would be expected in the clinical situation. Propafenone inhibited the HCN4 channel current
with an IC₅₀ value of 14.3 μM, but this was higher than the therapeutic concentration. The inhibitory effects of quinidine, disopyramide, cibenzoline, lidocaine, mexiletine, aprindine, propafenone, flecainide, propranolol, and verapamil on the HCN4 channel current were weak at their respective therapeutic concentrations, exhibiting IC₅₀ values of 78.3, 249, 46.8, 276, 309, 43.7, 14.3, 1700, 50.5, and 44.9 μM, respectively. Since the calculated IC₅₀ value for these antiarrhythmic drugs in inhibiting the HCN4 channel current was much higher than their therapeutic concentrations (21, 28), the inhibitory effects of these drugs on Iᵦ in the clinical setting would be small.

d,l-Sotalol hardly affected the HCN4 channel current. Among the class I, II, III, and IV drugs examined in this study, amiodarone showed the most potent inhibitory effect on the HCN4 channel current. It has been acknowledged that Iᵦ plays an important role in producing abnormal automaticity from the ectopic focus. Especially when the inward rectifier K⁺ current is suppressed by a decrease in extracellular K⁺ concentration (hypokalemia) or the sympathetic nerve system is activated, automatic activity from cardiac tissues other than the SA node (such as Purkinje fibers) may be accelerated. In addition, the Iᵦ densities in left ventricular myocytes were reportedly increased in hypertrophied hearts or end-stage failing hearts, leading to an increased propensity of ventricular arrhythmias (12 – 14). Antiarrhythmic drugs inhibiting the HCN4 channel current may suppress ectopic automaticity arising from phase 4 depolarization. In our preliminary experiments the isoproterenol (3 nM)-induced automaticity from isolated rat ventricular tissues were effectively suppressed by 10 μM bepridil, but not by 30 μM mexiletine (unpublished observations). These preliminary data seem to be consonant with the potencies of the antiarrhythmic drugs in inhibiting the HCN4 channel current, shown in this study. A recent study showed that paroxysmal atrial fibrillation may be triggered from ectopic firing foci located in the pulmonary veins (29). From the morphology of the action potentials recorded from pulmonary veins (30), a slow diastolic depolarization appears to be involved in the genesis of the spontaneous activity. Indeed, when immunostaining of the rat atrium-pulmonary vein tissues was conducted using an anti-HCN4 antibody, positive

**Fig. 7.** Inhibitory effects of class IV drugs, verapamil and bepridil, on HCN4 channel current in HEK293 cells. Representative current traces before and after verapamil (30 μM) or bepridil (10 μM) are indicated on the left side. The voltage protocol is indicated in the inset. Current–voltage relationships for the fully activated channel currents measured at the end of 2000-ms hyperpolarizing pulses before and after class IV drugs are indicated in the right panels. Concentration–response curves for the inhibitory effects of verapamil and bepridil on HCN4 channel current are also shown on the right. Percent inhibition of the current after a hyperpolarizing pulse to −70 mV was used as an index of block, and the IC₅₀ values were calculated. Values are each the mean ± S.E.M. of 5 – 6 experiments. *P<0.05 vs. control value.
staining for HCN4 channel proteins was observed at the boundary of rat atrium and pulmonary veins, as well as the SA node (31). In addition, both amiodarone and zatebradine suppressed the spontaneous activity observed in isolated rat pulmonary vein–atrial preparations (31). Therefore, antiarrhythmic drugs inhibiting HCN4 channel current may suppress the spontaneous activity from myocardial sleeves of pulmonary veins by inhibiting $I_f$. On the other hand, the antiarrhythmic drugs inhibiting HCN4 channels may cause sinus bradycardia since the channels abundantly distribute in the sinoatrial node region. Therefore, the antiarrhythmic drugs with potent inhibitory action on HCN4 channels should be administered to the patients with sinoatrial node dysfunction with great caution. It is noteworthy that both amiodarone and bepridil inhibit $Na^+/Ca^{2+}$ exchange current, which may also affect pacemaker function (32, 33).

There are several limitations in this study. First, subunit stoichiometry of HCN channels in the heart has not been established. In this study, only the effects of antiarrhythmic drugs on the homomeric tetramer of HCN4 channels were evaluated. If native $I_f$ channels are composed of HCN4 and HCN1/HCN2 channels with or without accessory $\beta$-subunit, drug sensitivity may be altered. Second, it is not known from this study how much HCN4 channel inhibition may be needed to suppress automatic activity caused by phase 4 depolarization. Third, the therapeutic concentration and the calculated IC$_{50}$ value of each antiarrhythmic drug for inhibiting the HCN4 channel current were compared without taking the protein binding of the drug into consideration. However, information about the effects of many antiarrhythmic drugs on the HCN4 channel, a dominant isoform of the HCN channels in the heart, may be useful for the treatment of various arrhythmias without untoward effects.

In conclusion, amiodarone and bepridil, multichannel blocking antiarrhythmic drugs, potently inhibited the HCN4 channel current. The inhibitory effects of quinidine, disopyramide, cibenzoline, lidocaine, mexiletine, aprindine, propafenone, flecaïnide, propranolol, and verapamil on the HCN4 channel current were weak in their therapeutic concentrations and $d,l$-sotalol hardly affected the current. Information about the effects of many antiarrhythmic drugs on the HCN4 channel may be useful for selecting an appropriate drug for treatment of each type of arrhythmia and at the same time, minimizing the adverse effects.

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