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SPECIAL REPORT

# Gadolinium inhibits $Na^+$-$Ca^{2+}$ exchanger current in guinea-pig isolated ventricular myocytes

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The trivalent cation, gadolinium ($Gd^{3+}$) is commonly used to inhibit stretch-activated channels. In this report, we show that $Gd^{3+}$ also inhibits ionic current ($I_{NaCa}$), carried by the $Na^+$-$Ca^{2+}$ exchanger protein. Under selective recording conditions, $Gd^{3+}$ inhibited both outward and inward $I_{NaCa}$ from guinea-pig isolated ventricular myocytes in a dose-dependent manner, with half-maximal inhibition concentrations ($IC_{50}$) of $30.0 \pm 4.0$ $\mu$M at $+60$ mV (Hill-coefficient, $h = 1.04 \pm 0.13$) and $20.0 \pm 2.7$ $\mu$M at $-100$ mV ($h = 1.13 \pm 0.16$), respectively ($P > 0.05$, $n = 5-9$). Thus, inhibition was not voltage-dependent. The time from $Gd^{3+}$ application to steady-state effect was slow compared to the divalent blocker $Ni^{2+}$. The slow time course appeared to reflect gradual $Gd^{3+}$ accumulation at its binding site on the exchanger, rather than a use-dependent blocking mechanism. This study indicates that for experiments in which $Gd^{3+}$ is used, its inhibitory effect on $I_{NaCa}$ should be taken into account.
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**Keywords:** $Na^+$-$Ca^{2+}$ exchanger; gadolinium ($Gd^{3+}$); ventricular myocyte

**Abbreviations:** $E_{rev}$, reversal potential; $Gd^{3+}$, gadolinium; $I_{Ca,L}$, L-type calcium current; $I_{Kr}$, rapid delayed rectifier current; $I_{NaCa}$, $Na^+$-$Ca^{2+}$ exchanger current; SAC, stretch-activated channel

**Introduction** Gadolinium ($Gd^{3+}$) is a trivalent lanthanide that has been widely used as a stretch-activated channel (SAC) blocker in a wide range of tissue types (Yang & Sachs, 1989). Some reports, however, indicate that the actions of $Gd^{3+}$ are not restricted to SACs. In ventricular cardiomyocytes, $Gd^{3+}$ blocks L-type Ca current ($I_{Ca,L}$; Lacampagne *et al*., 1994) and the rapid delayed rectifier K current ($I_{Kr}$; Pascarel *et al*., 1998), whilst not inhibiting stretch-induced increase of intracellular $Ca^{2+}$ (Hongo *et al*., 1997) or SAC in atrial cells (Zhang *et al*., 2000). Hongo *et al*. (1997) suggested that the lack of effect of $Gd^{3+}$ on stretch-induced increases in $[Ca^{2+}]_i$ may result from an additional action: $Gd^{3+}$ might inhibit $Ca^{2+}$ extrusion on the sarcolemmal $Na^+$-$Ca^{2+}$ exchanger, as this plays a vital role in $Ca^{2+}$ homeostasis (Blaustein & Lederer, 1999).

The exchanger is expressed widely in mammalian tissues e.g. heart, brain, kidney, colon, spleen, lung and pancreas (Blaustein & Lederer, 1999). Thus if $Gd^{3+}$ can block the exchanger this would be of relevance to studies on a variety of experimental systems. The stoichiometry of the $Na^+$-$Ca^{2+}$ exchange process ($3Na^+$ ions transported per 1 $Ca^{2+}$ ion) means that it generates an ionic current ($I_{NaCa}$; Kimura *et al*., 1986; 1987). The aim of the present study was to utilize direct measurements of $I_{NaCa}$ from guinea-pig ventricular myocytes to determine whether or not $Gd^{3+}$ does inhibit the exchanger.

**Methods** *Cell isolation* Single ventricular myocytes from hearts of male guinea-pigs (400–600 g) were isolated as previously described (Hobai *et al*., 1997). The cells were kept at 4°C in high-$K^+$, low $Cl^-$ storage medium (Kraft-Brühe, KB medium) containing (in mM): L-glutamate 100, KCl 30, Na-pyruvate 5, taurine 20, creatine 5, succinic acid 5, $Na_2ATP$ 2, $\beta$-OH butyrate 5, glucose 20, $MgCl_2$ 5, EGTA 1, HEPES 10 (pH adjusted to 7.2 with KOH).

*Voltage-clamp technique* An aliquot of cell suspension was allowed to settle on the glass bottom of a Perspex chamber on the stage of an inverted microscope (Nikon Diaphot) for several minutes. Then, the bath was continuously superfused at 37°C with Tyrode's solution (see below). Whole-cell patch clamp experiments were performed using an Axopatch 200A amplifier (Axon instruments, U.S.A.). Patch pipettes (Corning 7052 glass, AM Systems) were pulled using a Flaming/Brown P87 puller and fire-polished to final resistance of 1–2 MΩ (Narishige MF 83 microforge, Japan). Protocols were generated and data recorded on-line with p-Clamp 6.0 software *via* an analogue-to-digital converter (Digidata 1200B, Axon instruments, U.S.A.). Data are expressed as mean ± s.e.mean; *t*-test and ANOVA were used for statistical analysis. *P* values less than 0.05 were taken as significant.

*Solutions* Tyrode's solution contained (in mM): NaCl 140, HEPES 5, glucose 10, KCl 4, $CaCl_2$ 2.5, $MgCl_2$ 1 (pH adjusted to 7.45 with NaOH). We used similar solutions to Hinde *et al*. (1999) to measure $I_{NaCa}$. The extracellular solution for recording $I_{NaCa}$ was $K^+$-free Tyrode's solution containing 10 $\mu$M strophanthidin, 10 $\mu$M nifedipine and 1 mM $BaCl_2$ to eliminate K, Ca, background and Na-K pump currents. Solutions were applied using a home-built rapid solution application device. Intracellular solution contained (in mM): Cs-aspartate 113, NaCl 20, $MgCl_2$ 0.4, Tris-ATP 5, HEPES 10, glucose 5, BAPTA 10, tetraethylammonium (TEA) 20, $CaCl_2$ 1, pH 7.2 (titrated with CsOH). The combination of 10 BAPTA and 1 $CaCl_2$ gave a free pipette Ca concentration of 20 nM (calculated with the Maxchelator program).

*Chemicals* Gadolinium chloride was purchased from Sigma and dissolved in the external $I_{NaCa}$ solution to the concentrations shown in 'Results'.

**Results** The solutions described above have previously been demonstrated to allow $I_{NaCa}$ to be measured as current sensitive to external $Ni^{2+}$ or 0Na/0Ca solution (Hinde *et al*., 1999). To determine the effects of $Gd^{3+}$, we applied descending

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ramp pulses every 12 s from +80 mV to −120 mV (dV/dt = 0.4 V $s^{-1}$) from a holding potential of −80 mV. Representative net currents before and after $Gd^{3+}$ are shown in Figure 1A inset. Both outward and inward currents were inhibited by 100 $\mu$M $Gd^{3+}$. The $Gd^{3+}$-sensitive current showed an outwardly rectifying current-voltage (I−V) relationship with a reversal potential ($E_{rev}$) close to −60 mV (Figure 1A). The observed $E_{rev}$ was higher than that calculated for $I_{NaCa}$ using the free [Ca] and [Na] values for the pipette solution given in the Methods (which was in excess of −150 mV, using equation 6′ of Blaustein & Lederer, 1999). It was, however, consistent with recent data (Convery & Hancox, 1999), which suggest that buffering of subsarcolemmal and bulk [Ca] can differ under selective recording conditions for $I_{NaCa}$. The characteristics of the $Gd^{3+}$-sensitive current, therefore, resembled those reported previously for $I_{NaCa}$ (e.g. Convery & Hancox, 1999; Hinde *et al*., 1999). To confirm the identity of the $Gd^{3+}$-sensitive current, extracellular $Na^+$ and $Ca^{2+}$ were replaced with $Li^+$ and $Ba^{2+}$ (0Na/0Ca solution to abolish $I_{NaCa}$). With the exchanger inhibited, the residual current was not significantly affected by $Gd^{3+}$ (Figure 1B). Similar results were obtained when $I_{NaCa}$ was completely blocked with the widely used blocker of $I_{NaCa}$ $Ni^{2+}$ (10 mM; data not shown). Collectively, these data showed that the current component inhibited by $Gd^{3+}$ was $I_{NaCa}$.

The sensitivity of $I_{NaCa}$ to $Gd^{3+}$ was concentration-dependent within the concentration range tested of 2−200 $\mu$M. Figure 2A shows the effect of three different [$Gd^{3+}$] on the I-V relationship for $I_{NaCa}$ ($n$ = 6−9 cells for these three concentrations). Similar suppression of both outward and inward current amplitudes was observed at particular [$Gd^{3+}$]. Figure 2B shows the current amplitudes at +60 mV and −100 mV plotted against [$Gd^{3+}$] between 2−200 $\mu$M. Half-maximal inhibition was observed at $Gd^{3+}$ concentrations ($IC_{50}$) of 30.0±4.0 $\mu$M at +60 mV (Hill-coefficient, h was 1.04±0.13, $n$ = 5−9) and 20.0±2.7 $\mu$M at −100 mV (h was 1.13±0.16, $n$ = 5−9). These values were not significantly different ($P$ > 0.05). The similar $IC_{50}$ values suggest that the inhibition of $I_{NaCa}$ by $Gd^{3+}$ was not voltage-dependent. The Hill-coefficient close to 1 suggests that $Gd^{3+}$ exerted its inhibitory effect by at least one molecule of $Gd^{3+}$ binding to one exchanger protein molecule.

In contrast to its effects on $I_{Ca,L}$ (Lacampagne *et al*., 1994) or $I_{Kr}$ (Hongo *et al*., 1997), $Gd^{3+}$ was slow to exert its full effect on $I_{NaCa}$. Application of $Gd^{3+}$ (100 $\mu$M) for up to 2 min only partially suppressed $I_{NaCa}$ while the time for its effect to reach the steady-state was over 6 min. The slow time course of inhibition was not attributable to the perfusion system as (a) $Ni^{2+}$ almost completely suppressed $I_{NaCa}$ within 12 s, (b) 100 $\mu$M $Gd^{3+}$ completely blocked $I_{Ca,L}$ within only seconds (data not shown). These observations were consistent with previous reports (Lacampagne *et al*., 1994; Hinde *et al*., 1999). In addition, reversal of inhibition was rapid on switching to $Gd^{3+}$-free solution. For 100 $\mu$M $Gd^{3+}$, the current attained 44.6±5.5% ($n$ = 6) of the control value within 12 s of washout. This did not increase with repetitive pulsing; thus, under our conditions reversibility was incomplete. The time-course of $Gd^{3+}$ inhibition of the reverse and forward modes of the exchanger (outward and inward current, respectively) was ascertained by normalizing the mean outward and inward current amplitudes at +60 mV and −100 mV and plotting amplitude relative to control against duration of $Gd^{3+}$

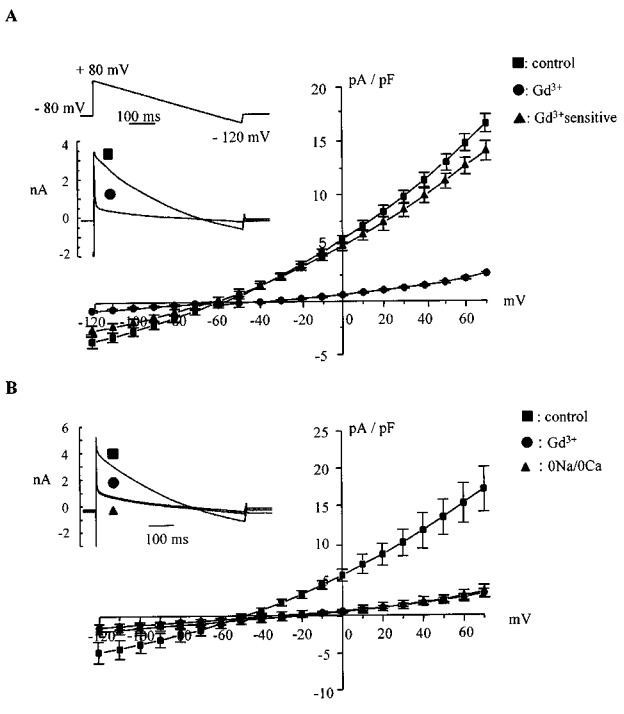


**Figure 1** The effect of 100 $\mu$M $Gd^{3+}$ on $I_{NaCa}$. (A) Current-voltage (I-V) relationship of current sensitive to external $Gd^{3+}$, obtained by subtracting from control the current after 100 $\mu$M $Gd^{3+}$. Inset shows the pulse protocol and representative currents. (B) The effects of $Gd^{3+}$ under conditions in which $I_{NaCa}$ was abolished by 0Na/0Ca solution. Current in the presence of 0Na/0Ca (minus $Gd^{3+}$) was shown for comparison. $Gd^{3+}$ did not inhibit residual current. Inset shows representative currents.

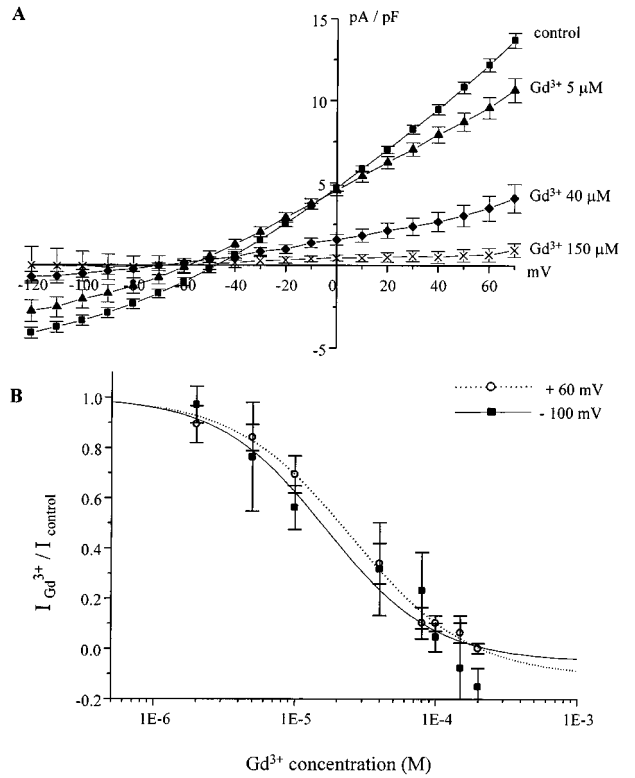


**Figure 2** Dose- and voltage-dependence of $Gd^{3+}$ inhibition of $I_{NaCa}$. (A) Mean I-V relationship of current amplitudes at different $Gd^{3+}$ concentrations. $Gd^{3+}$ subsequently suppressed $I_{NaCa}$. (B) Dose-response curves for the effect of $Gd^{3+}$ on $I_{NaCa}$. Currents were measured at +60 mV and −100 mV. Continuous curves were plotted according to the Michaelis-Menten equation: $I_{Gd^{3+}}/I_{control} = 1/(1+([Gd^{3+}]/IC_{50})^h)$. h is Hill coefficient.

exposure (Figure 3). The decline of both inward and outward $I_{NaCa}$ was fitted best by a bi-exponential function. The initial decline was slightly faster at +60 mV than at −100 mV; but this difference was not significant ($P>0.05$). The slower phase of decline was similar at the two potentials.

$Gd^{3+}$-inhibition of calcium current has been reported to depend on the extent of channel activation (Biagi *et al*., 1990). It seemed possible, therefore, that the slow time-course with which $Gd^{3+}$ exerted its steady-state effect might have reflected dependence of the inhibition on repeated activation (use-dependence) of the exchanger. The following approach was used to investigate this. First, the net 'control' $I_{NaCa}$ was obtained by measuring the current sensitive to $Ni^{2+}$ (10 mM) application. In seven cells, current was monitored for 5 min during continuous pulsing. In five cells, the control $I_{NaCa}$ was obtained as above, and then $Gd^{3+}$ (100 $\mu$M) applied: cells were exposed to $Gd^{3+}$ for 5 min, but in the presence of 0Na/0Ca solution to immobilize the exchanger and without application of test pulses. (Figure 4, rested in 0Na/0Ca solution). If the block depended on repeated activation of the exchanger, we reasoned that on readmitting external Na and Ca after 5 min (in the presence of $Gd^{3+}$), relatively little block would be expected on the first pulse, but would increase with repeated stimulation. In fact, a large reduction of the current amplitude was observed on the first pulse with this method, to an extent that was not significantly different ($P>0.05$) from that with continuous pulsing. Similar results were also observed with another approach (to measure the $E_{rev}$ for $I_{NaCa}$ for each cell and then hold the membrane potential at this value in the presence of $Gd^{3+}$ (100 $\mu$M) for 5 min; the $I_{NaCa}$ elicited by the first ramp after this period was then measured). These data suggested that the time course of the effect of $Gd^{3+}$ could not be accounted for by slow use-dependence of the inhibition.

**Discussion** Whilst there has been no previous study that has shown directly inhibition of the sarcolemmal $Na^+$-$Ca^{2+}$ exchanger by $Gd^{3+}$, computer-simulations by Hongo *et al.* (1997) led them to suggest that the blockade of the exchanger was required to reproduce experimental data showing a stretch-induced increase in guinea-pig ventricular $[Ca^{2+}]_i$. Earlier data had suggested that the trivalent cation $La^{3+}$ can inhibit the $Na^+$-$Ca^{2+}$ exchanger (Kimura *et al*., 1986), but direct information regarding $Gd^{3+}$ has not until now been available. Our data show that $Gd^{3+}$ inhibits the cardiac $Na^+$-$Ca^{2+}$ exchanger with a potency that compares favourably with other divalent or trivalent inorganic inhibitors: e.g. $IC_{50}$ for $Ni^{2+}$ of 0.3 mM (Hinde *et al*., 1999) and inhibition by concentrations >0.25 mM $La^{3+}$ in smooth muscle cells (Shimizu *et al*., 1997). The Hill co-efficient values near 1 are consistent with observations on other inorganic inhibitors (Blaustein & Lederer, 1999).

The blocking effect of $Gd^{3+}$ on the $Na^+$-$Ca^{2+}$ exchanger was found to be voltage-independent, but was dependent on the duration of exposure to $Gd^{3+}$, with block increasing to a steady-state value over a period of minutes after initial application. These observations appear to exclude a surface charge mechanism (which would be expected to show voltage dependence) but are most consistent with $Gd^{3+}$ gradually accumulating at a site that is accessible from the external surface of the cell. On the basis of the experiments shown in Figure 4 it seems reasonable to conclude that the inhibition does not depend on the exchanger being repeatedly activated (in order to facilitate $Gd^{3+}$ binding), as similar levels of block were observed with exchanger immobilization as with repeated activation. Additionally, preliminary data suggested that $Gd^{3+}$ might not be able to readily reach its binding site from the cell interior, because $Gd^{3+}$ in the pipette dialysate (at concentrations up to 5 mM) showed little block of $I_{NaCa}$ (data not shown). Intriguingly, the trivalent cation $La^{3+}$ has been reported to enter cells (Shimizu *et al*., 1997; Peeters *et al*., 1989), and therefore might possibly act at the inside of the plasma membrane.

The demonstration that $Gd^{3+}$ blocks $I_{NaCa}$ is important both because the exchanger is present in a range of tissue types, and because $Gd^{3+}$ is a widely used experimental tool, particularly in studying stretch-related responses. When interpreting its effects in various experimental systems, potentially diverse actions should be considered (Caldwell *et*

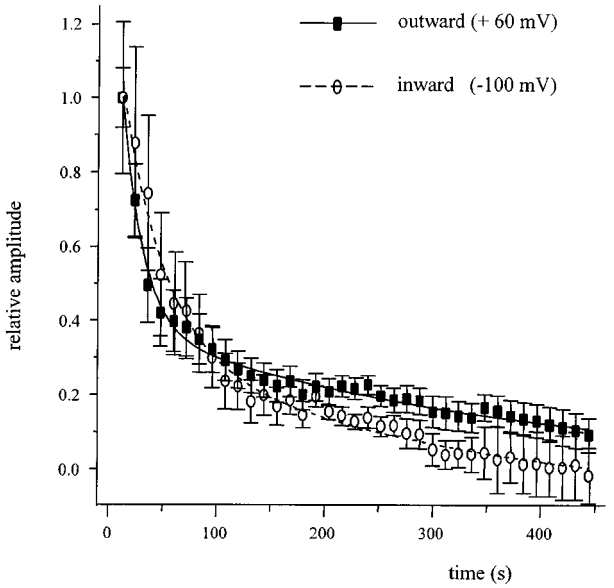


**Figure 3** Time-dependence of $Gd^{3+}$ inhibition of $I_{NaCa}$. Proportion of control $I_{NaCa}$ after $Gd^{3+}$ was applied at +60 mV and −100 mV plotted against time. Both data sets were fitted with a double exponential function: $\tau_1=19.2\pm0.06$ s, $\tau_2=347.4\pm21.3$ s at +60 mV and $\tau_1=38.6\pm4.3$ s, $\tau_2=303.0\pm36.5$ s at −100 mV, $n=7$.

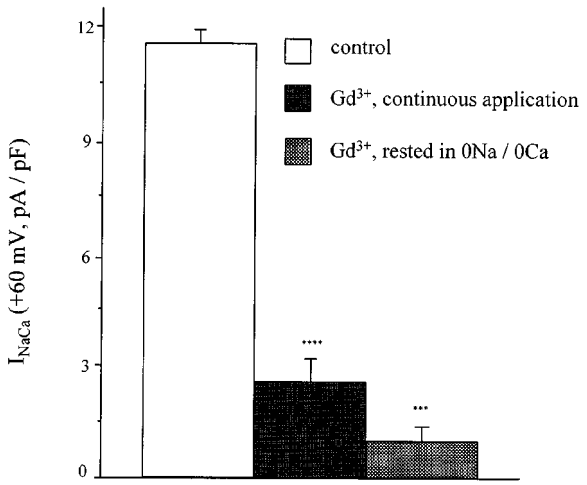


**Figure 4** Lack of use-dependence of the effect of $Gd^{3+}$ on $I_{NaCa}$. $I_{NaCa}$ amplitudes at +60 mV under three conditions were compared: Control; 100 $\mu$M $Gd^{3+}$ was continuously perfused for 5 min during the time $I_{NaCa}$ was repetitively activated by continuous pulsing ($n=7$); Extracellular $Na^+$ and $Ca^{2+}$ were replaced by equimolar $Li^+$ and $Ba^{2+}$ (0Na/0Ca solution, $n=5$) during 5 min of $Gd^{3+}$ exposure, then $I_{NaCa}$ measured when Na and Ca were readmitted. (****,$P<0.00002$ with respect to control $I_{NaCa}$; ***$P<0.0007$ with respect to control $I_{NaCa}$).

*al*., 1998). To these, it would appear now necessary to add its potent inhibitory effects on $I_{NaCa}$. Further, it is possible that this action of $Gd^{3+}$ may help increase understanding of the range of roles played by the $Na^+$-$Ca^{2+}$ exchanger in both physiological and pathological situations.

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