Study of transmembrane La^{3+} movement in rat ventricular myocytes by the patch-clamp technique

YANG Pin, DU Huizhi & XUE Shaowu

Institute of Molecular Science, Shanxi University, Taiyuan 030006, China

Correspondence should be addressed to Yang Pin (e-mail: yangpin@sxu.edu.cn)

Abstract **We have studied transmembrane La^{3+} movement in rat ventricular myocytes for the first time by using the whole-cell patch-clamp recording mode. La^{3+} (0.01—5.0 mmol/L) could not bring out inward currents through the L-type calcium channel in rat ventricular myocytes, while it could enter the cells by the same way carried by 1 μmol/L ionomycin. When the outward Na^+ concentration gradient is formed, La^{3+} can enter the cells via Na-Ca exchange, and the exchange currents increase with the increase of external La^{3+} concentrations. But compared with Na-Ca exchange currents in the same concentration, the former is only 14%—38% of the latter. The patch-clamp experiment indicates that La^{3+} normally can not enter ventricular myocytes through L-type calcium channel, but it can enter the cells via Na-Ca exchange.**

Keywords: whole-cell patch-clamp recording, ventricular myocyte, L-type calcium channel, Na-Ca exchange, La^{3+}, Ca^{2+}.

In recent years, rare earths have been widely applied in the fields of agriculture, livestock husbandry and medicine. More and more rare earths have entered the environment, even the food chain. Although much work has been carried out on the toxicological research, the action mechanism of rare earths on animals and plants is not clear, and the research at the ion channel level has just been started. Whether rare earths can enter cells to participate in the physiological metabolize of animals and plants is very important to illuminate the biological effects and the action mechanisms of rare earths on animals and plants[1]. L-type calcium channel and Na-Ca exchanger are the main paths of the transmembrane Ca^{2+} movement in the rat ventricular myocyte membrane[2—4]. La^{3+} is very similar to Ca^{2+} in chemical properties[5], and Ca^{2+} can enter into the cells by the two paths. But can La^{3+} also enter the cells by the same way? Original research showed that La^{3+} could only bind to the calcium-channel binding site which had high affinity to high charge ions, but it could not enter the cells[6]. Recently, by using the fluorescence technique, many experts both in China and abroad discovered that La^{3+} could enter ventricular myocytes and chromaffin cells via Na-Ca exchange[7—8]. Compared with the fluorescence technique, the patch-clamp technique can record the change of ion-channel currents on single cell membrane with the current sensitivity of 1 pA, space resolving power of 1 μm and time resolving power of 10 μs. Therefore, it has very high reliability[9,10]. After constant improvements of more than 20 years, the patch-clamp technique has been widely applied in the life science research field. By means of repatch, it becomes available to monitor the long-term effect of reagents at different concentrations on ion channel in the same cell membrane. We studied the transmembrane La^{3+} movement in rat ventricular myocytes for the first time by using the patch-clamp recording mode, and illustrated the mechanism of transmembrane movement of La^{3+}.

1 Materials and methods

(i) Preparation of single ventricular myocyte. Single ventricular myocyte was isolated from Wistar rats (250—300 g, male or female, from the Animal House of the Institute of Radiation Prevention, the Chinese Academy of Sciences) as previously described[11]. In brief, hearts were quickly moved and mounted on a Langendorff apparatus for retrograde perfusion at 37℃, firstly with free-Ca^{2+} Tyrode's solution for 5 min, then with slow-Ca^{2+} solution containing 0.03% collagenase (Ⅰ), 0.25% Taurine and 50 μmol/L Ca^{2+}. Afterwards, the hearts were incubated in KB solution (100% O_2 saturated) and minced and dispersed with a pipette. The suspension was filtered through the 190 μm nylon mesh, and kept at room temperature for 4 h before use.

(ii) Patch-clamp recording. The whole-cell patch-clamp recording mode adopt Hamill's patch-clamp methods[10]. Patch-clamp pipettes (1—2 μm) were prepared from glass capillaries (BJ-40, diameter (1.5±0.1) mm, Beijing), and pulled on a multi-stage programmable puller (NATISHZGE, Japan). Before the experiment, they were filled with internal solutions (pipette resistance 2—5 MΩ), then mounted on the holder which connected the Ag-AgCl reference electrode, and then screwed. The cell suspensions were put in a chamber mounted on the stage of an inverted microscope. After being settled to the bottom of the chamber for 15 min, cells were superfused with external solutions at the rate of 2 mL/min for 30 min to wash the cell surfaces. After another 30 min, only the cells with rod-shape and clear striations were used in experiments. Giga-Ω seals between the electrode and the cell membrane (above 2 GΩ) were obtained by gentle suction. After break of the membrane, the whole-cell recording mode was formed. In the voltage-clamp mode, we recorded I_{Ca}, $I_{Na/Ca}$ and so on. Currents were amplified by an amplifier (Digidata 1200), and the Pclamp 6.0.4 software was used to produce protocols, acquire and process data. Data were analyzed and then figures were plotted with Clampfit and MICROCAL-ORIGIN (5.0).

(iii) Preparation of experimental solutions (mmol/L). Free-Ca^{2+} Tyrode's solution: NaCl 125, $MgCl_2$ 1, KCl 5.4, NaH_2PO_4 0.33, HEPES 10, Glucose 10, pH adjusted to 7.33 with NaOH. Improved Tyrode's solution: LiCl 125, $MgCl_2$ 1, KCl 5.4, NaH_2PO_4 0.33, HEPES 10, Glucose 10, pH adjusted to 7.33 with CsOH. KB solution: KCl 70,

KH_2PO_4 20, $MgCl_2$ 5, Glucose 10, HEPES 10, EGTA 0.5, Taurine 20, *L*-Glutamic acid 50, pH adjusted to 7.33 with KOH. Internal solution 1 used to record $I_{Ca:}$ CsCl 140, NaCl 1, $CaCl_2$ 1, MgATP 3, EGTA 11, HEPES 10, pH adjusted to 7.32 with KOH. Internal solution 2 used to record $I_{Na\text{-}Ca}$: CsCl 104, TEACl 20, NaCl 30, EGTA 10, HEPES 5, MgATP 3, $CaCl_2$ 0.1, pH adjusted to 7.32 with CsOH.

2 Results

(i) Whether La^{3+} enters ventricular myocytes through L-type calcium channel

(1) Normal L-type calcium currents. Internal solution 1 and the external solution included Free-Ca^{2+} Tyrode's solution with 1.8 mmol/L Ca^{2+} were used in the experiment. When the holding potential was – 40 mV, the cell was depolarized to + 40 mV for 500 ms at frequency of 1 Hz (step +10 mV). Then we acquired inward current curves (fig. 1(a)). Fig. 1(b) shows the current-voltage relationship. Every 2 min current signals were recorded, and then drugs were not added in external solutions until current signals decline. 0.5 μmol/L BAYK8644 firstly made the current peak increase, which basically resumed after cells were washed out. Then 20 μmol/L verapamil made the current peak decrease, which basically resumed after cells were washed out. These signals were from the same cell. Fig. 2 shows the current relationship with the membrane potential of +10 mV. In the experiment, Cs^+ in pi- pette solutions blocked the K^+ current; the holding potential was kept at – 40 mV to inactivate the Na^+ channel and the T-type calcium channel. The calcium-channel agonist BAYK8644 made the current peak increase, while the calcium-channel blocker verapamil made the current peak

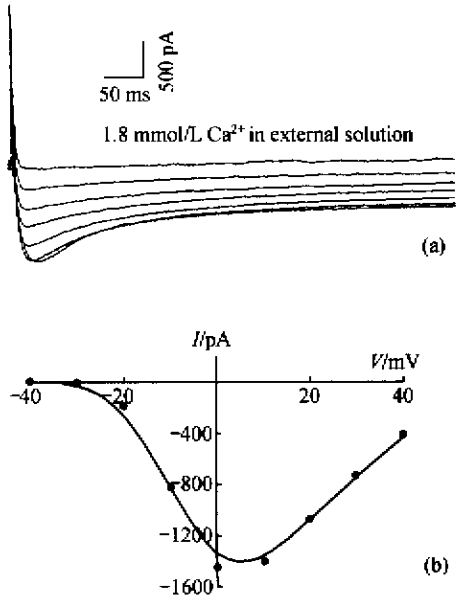

Fig.1. L-type calcium-channel current (a) and current-voltage relationship curve (b).

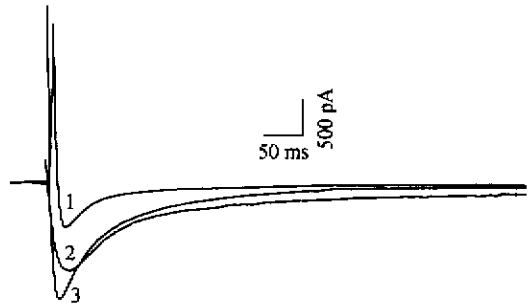

Fig. 2. Effect of 0.5 μmol/L BAYK8644 (3) and 20 μmol/L verapamil (1) on L-type calcium-channel currents. Curve 2 is control.

decrease. These results indicate that the current signals are L-type calcium-channel currents[12,13]. We often observed the run-down of calcium currents, which might have something to do with the increase of the cellular calcium concentration and the diffusion of cytoplasm ingredient to the pipette[13]. In the experiment, 3 mmol/L ATP and 11 mmol/L EGTA were added to the internal solution to decrease the run-down degree, and ensure the reliability of the experiment.

(2) The effect of ionomycin on L-type calcium-channel currents: Internal solution 1 and the external solution included Free-Ca^{2+} Tyrode's solution with 1.8 mmol/L Ca^{2+} were used in the experiment. The patch-clamp parameters were set as above. Curve 1 in fig. 3 is the normal L–type calcium-channel current as reference. Curve 2 in fig. 3 is the current signal in the same cell after adding 1 μmol/L ionomycin into the external solution. According to fig. 3, calcium currents increased from (700 ± 5) pA to (2000 ± 5) pA, and the increase rate is up to 185.7% ($n > 5$). Ionomycin is a kind of Ca^{2+} carrier like a chain, involving two furan rings. It has an alcohol hydroxyl at one end of the chain and a carboxyl at the other end. In normal physiological conditions, ionomycin has the negative charge because of the dissociation of the carboxyl. When ionomycin coordinates with the metal ion, the hydrogen on the alcohol hydroxyl combines with the oxygen with the negative charge on the carboxyl. Therefore hydrophobic groups are at outward of the coordinate compounds and the whole molecule is liposoluble, which pass the lipoid-bilayer of the cell membrane and releas the metal ion into the cell. Ionomycin can coordinate 1∶1 with Ca^{2+} and transport the Ca^{2+} into the cell, which leads to the increase of the calcium current[14].

(3) The inhibitory effect of La^{3+} on L-type calcium-channel currents. Internal solution 1 and the external solution included Free-Ca^{2+} Tyrode's solution with 1.8 mmol/L Ca^{2+} were used in the experiment. The patch-clamp parameters were set as above. Normal L-type calcium-channel currents were recorded as reference. When current signals were steady every 2 min, La^{3+} was added to the external solution in order of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5},

10^{-4}, 10^{-3}, 2×10^{-3} and 5×10^{-3} mol/L, then we recorded the corresponding current signals. Fig. 4 shows relationship curve of calcium-channel current vs. the logarithm of external La^{3+} concentration with the membrane potential of +10 mV. The data are the average of these currents (n = 5). According to fig. 4, when the external La^{3+} concentration is lower than 10^{-4} mol/L, the inhibitory effect is not obvious, while when the concentration is higher than 10^{-4} mol/L, the inhibitory effect evidently increases, and at the concentration of 5×10^{-3} mol/L, the currents are almost inhibited completely. La^{3+} is similar to Ca^{2+} in configuration, while La^{3+} has a bigger ion radius, higher charge density and higher affinity. Therefore, when La^{3+} at equivalent concentrations is added to the external solution containing Ca^{2+}, after competition against Ca^{2+}, La^{3+} is prior to combine with channel proteins, which brings about the deformation of the protein conformation, the block of the L-calcium channel and the inhibition of the currents. The result accords with the reference, and supports strongly the view that La^{3+} has the inhibitory effects on the calcium channel[15].

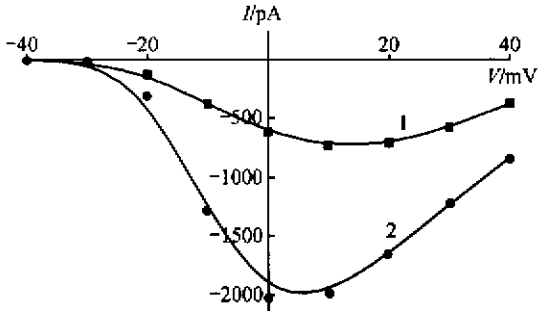

Fig.3. Effect of 1 μmol/L ionomycin (2) on L-type calcium-channel currents. Curve 1 is control.

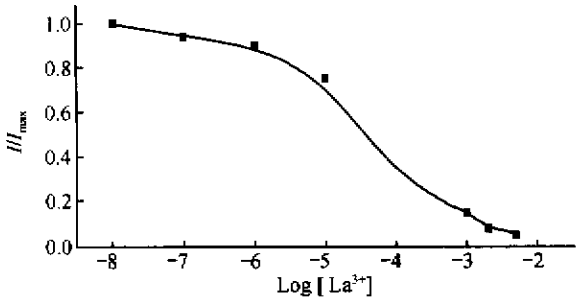

Fig. 4. Effect of La^{3+} at different concentrations in external solution on L-type calcium-channel currents.

(4) The action of La^{3+} on the ventricular-myocyte membrane. Internal solution 1 and the external solution included Free-Ca^{2+} Tyrode's solution with 0.1 mmol/L EGTA were used in the experiment. The patch-clamp parameters were set as above. Fig. 5(a) shows current signals after adding La^{3+} in order of 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 and 5.0 mmol/L into the external solution. Fig. 5(b) shows inward currents after sequentially adding 1 μmol/L ionomycin into the external solution. The same experiments were repeated (n > 5), and the results were similar. According to fig. 5, 1 μmol/L ionomycin can transport La^{3+} into ventricular myocytes and bring about inward currents with (500±5) pA, which is only 25% of the promoting effect of ionomycin on Ca^{2+}. The mechanism is similar to that of Ca^{2+}.

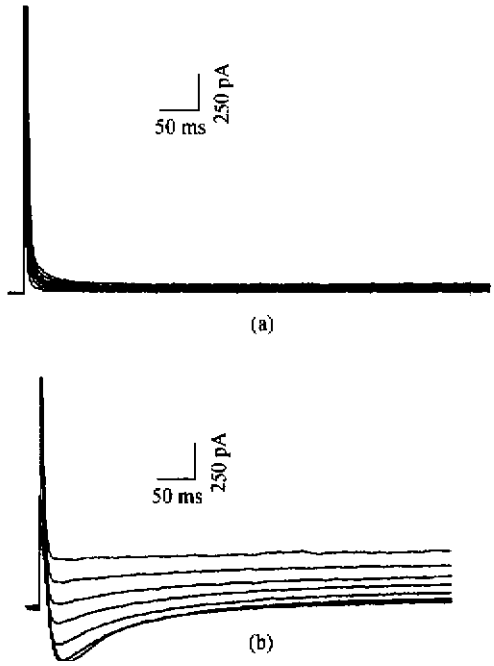

Fig. 5. Effect of 1 μmol/L ionomycin on transmembrane La^{+} movement in ventricular myocytes. (a) Control; (b) adding 1 μmol/L ionomycin in external solution.

(ii) Whether La^{3+} enters ventricular myocytes via Na-La exchange

(1) Ni^{2+} sensitive Na-Ca exchange currents. Internal solution 2 and external solution included improved Tyrode's solution together with 20 μmol/L ouabain, 1 mmol/L $BaCl_2$, 2 mmol/L CsCl, 20 μmol/L verapamil, and 0.1 mmol/L Ca^{2+} were used in the experiment. Ramp voltage-clamp pulses (+60 to −120 mV, 90 mV/s) were applied from a holding potential of − 40 mV. Normal Na-Ca exchange currents were recorded as reference. After the application of Ni^{2+} (5 mmol/L), the currents immediately decreased. The difference between currents in the absence and presence of Ni^{2+} (5 mmol/L) expresses the Ni^{2+}-sensitive Na-Ca exchange currents (fig. 6(a)). When current signals were steady every 2 min, Ca^{2+} was added to the external solution in order of 0.2, 0.5, 1.0, 2.0 and 5.0 mmol/L. Na-Ca exchange currents would increase with the increase of Ca^{2+} concentrations in the external solution (fig. 6(b), data were from the same cell). In the experiment, the Na^{+}-K^{+} pump, background currents, K^{+} channel and Ca^{2+} channel were blocked with 20 mol/L ouabain, 1

mmol/L $BaCl_2$, 2 mmol/L CsCl and 20 mol/L verapamil. In the internal solution, 20 mmol/L TEACl was also used to block the K^+ channel[16].

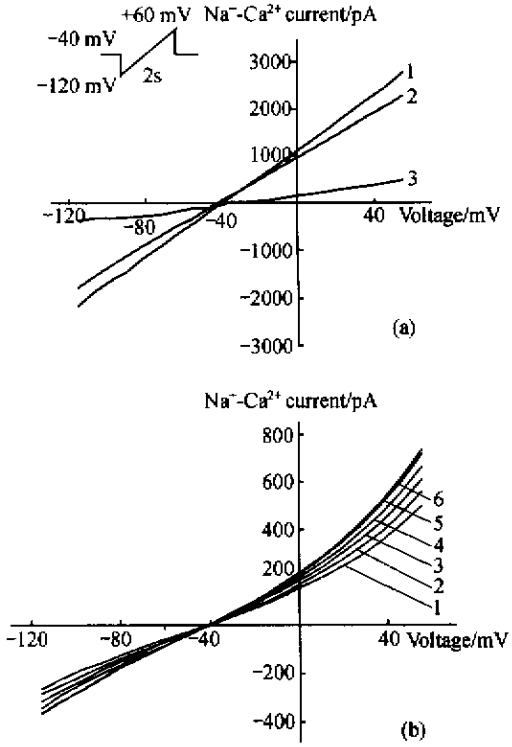

Fig. 6. Ni^{2+}-sensitive Na-Ca exchange current. (a) 1, Control; 2, adding 5 mmol/L Ni^{2+} in external solution; 3, 1–2, Ni^{2+}-sensitive Na-Ca exchange current. (b) Na-Ca exchange current with Ca^{2+} at different concentrations in external solution: 1—6, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mmol/L respectively.

(2) Ni^{2+}-sensitive Na-La exchange currents. Internal solution 2 and the external solution included improved Tyrode's solution together with 20 μmol/L ouabain, 1 mmol/L $BaCl_2$, 2 mmol/L CsCl, 20 μmol/L verapamil and 0.1 mmol/L EGTA were used in the experiment. Ramp voltage-clamp pulses (+ 60 to – 120 mV, 90 mV/s) were applied from a holding potential of – 40 mV. Current signals were firstly recorded in extra solution containing 0.01 mmol/L La^{3+}. After the application of Ni^{2+} (5 mmol/ L), the currents immediately decreased. The difference between currents in the absence and presence of Ni^{2+} (5 mmol/L) expresses the Ni^{2+}-sensitive Na-La exchange currents (fig. 7(a)). When current signals were steady every 2 min, La^{3+} was applied into the external solution in order of 0.2, 0.5, 1.0, 2.0 and 5.0 mmol/L. Na-La exchange currents would increase with the increase of La^{3+} concentrations in the external solution (fig. 7(b), data were from the same cell). However, the La^{3+} current was much less than the Ca^{2+} current in the same concentration, and the former was only 14%—38% of the latter (there was a tiny difference in data with different concentrations). The same experiments were repeated ($n > 5$) and the results were similar. Fig. 7 shows that when there is 0.01 mmol/L La^{3+} in the external solution, currents are every small. Because the change of the pure current caused by ion through the cell membrane is detected by the patch-clamp technique. 0.1 mmol/L EGTA in the external solution bind both the mineral Ca^{2+} permeating to external solution and a part of La^{3+}, which make the concentration of free La^{3+} very low. Therefore, currents are very small. When the concentration of La^{3+} is constantly increased, the concentration of free La^{3+} also increase, so currents also increase accordingly.

3 Discussion

The study on the L-type calcium channel shows that Ca^{2+} and channel proteins on the calcium binding site combine to form a kind of binding-protein which can activate the L-type calcium channel to make Ca^{2+} influx. When La^{3+} is added in the external solution, it coexists with minute quantity of Ca^{2+}. La^{3+} is prior to combine with channel proteins with its bigger ion radius, higher charge density and higher affinity, which brings about the deformation of protein conformation, the block of L-type calcium channel and the inhibition of transmembrane La^{3+} movement. When ionomycin is added in the external solution, it coordinates with La^{3+}, which makes the whole molecule liposoluble to carry La^{3+} into ventricular myocytes.

There is a kind of Na-Ca exchanger on ventricular myocyte membranes, which is a bidirectional carrier: Na^+ into the cell while Ca^{2+} out the cell; Ca^{2+} into the cell while Na^+ out the cell[17,18]. In the same experiment conditions, when La^{3+} substitutes for Ca^{2+} in external solutions, Na-La exchange currents similar to Na-Ca exchange currents appeares, which increase with the increase of external La^{3+} concentration. These results are similar to Na-Ca exchange currents. But compared to the latter, Na-La exchange currents are only 14%—38% of it, which is possibly because that La^{3+} has bigger ion radius, higher charge density and smaller diffusibility than Ca^{2+}. We deduce the action mechanism of this process as follows. La^{3+} is very similar to Ca^{2+} in configuration, ion radius and so on, so La^{3+} may be transported outward of the cell through the outward Na^+ concentration gradient by the exchanger. Similar to that of Na-Ca exchange, the carry mechanism may possibly be the "ping-pang" mode, which is supported by the patch-clamp technique and other techniques. That is to say, the transport of Na^+ is separated from that of La^{3+}. First the former combines with the carrier, and then the latter. The direction of transport is decided by the transmembrane electrochemical potential energy and Na^+ concentration gradient[19]. As to the proportion of the exchange, it still needs to be studied in detail.

This study of the mechanisms of transmembrane La^{3+} movement at ion channel level has some guiding significance to the further research of the effects of rare earths on physiological activities. Anyway, the action mechanism of rare earths to ventricular myocyte mem-

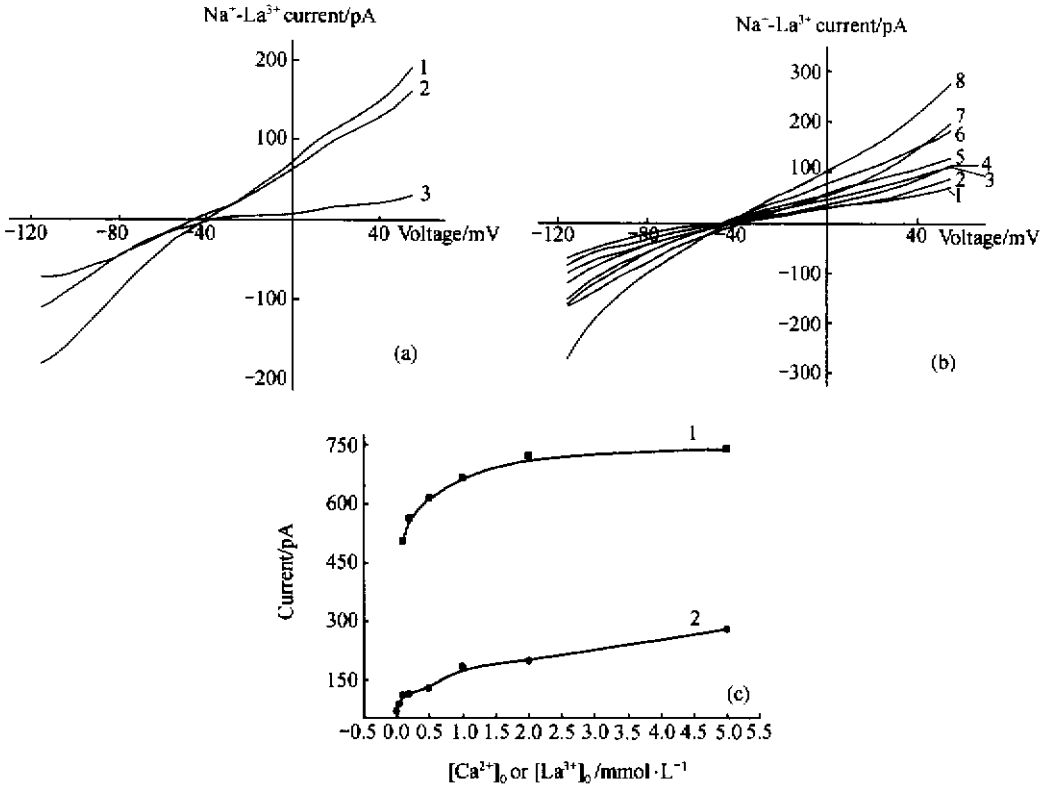

Fig. 7. Ni^{2+}-sensitive Na-La exchange current. (a) 1, Control; 2, adding 5 mmol/L Ni^{2+} in external solution; 3, 1–2, Ni^{2+}-sensitive Na-La exchange current. (b) Na-La exchange current with La^{3+} at different concentrations in external solution, 1—8, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mmol/L respectively.

brane is very complicate, which needs to be studied from different angles and with many different methods.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 29890280).

References

1. Evans, C. H., Biochemistry of the Lanthanides, New York and London: Plenum Press, 1990: 212—283.
2. Mogul, D. J., Fox, A. P., Evidence for multiple types of Ca^{2+} channels in acutely isolated CA3 neurons of the guinea-pig, J. Physiol., 1991, 433: 259.
3. Spedding, M., Paoletti, R., Classification of calcium channels and the sites of action of drugs modifying channel function, Pharmacol. Rev., 1992, 44: 363.
4. Barry, W. H., Smith, T. W., Mechanisms of transmembrane calcium movement in cultured chick embryo ventricular cells, J. Physiol., 1982, 325: 243.
5. Ni Jiazuan, Bioinorganic Chemistry of Rare Earth Elements (in Chinese), Beijing: Science Press, 1995, 2—4.
6. Peeters, G. A., Kohmoto, O., Barry, W. H., Detection of La^{3+} influx in ventricular cells by indo-1 fluorescence, Am. J. Physiol., 1989, 256 (Cell Physiol. 25): C351.
7. Reeves, J. P., Sutko, J. L., Sodium-calcium ion exchange in cardiac membrane vesicles, Proc. Natl. Acad. Sci., 1979, 76 (2): 590.
8. Powis, D. A., Clark, C. L., Obrien, K. J., Lanthanum can be transported by the sodium-calcium exchange pathway and directly triggers catecholamine from bovine chromaffin cells, Cell Calcium, 1994, 16: 377.
9. Neher, E., Sakamann, B., Single channel currents recorded from membrane of denervated frog muscle fibers, Nature, 1976, 260: 799.
10. Hamill, O. P., Marty, E., Neher, L. et al., Improved patch-clamp technique for high resolution current recording from cells and cell-free membrane patch, Pflugers. Arch., 1981, 391: 85.
11. Lu, J. Y., Wu, D. M., Wu, B. W., Na^+/Ca^{2+} exchange current in myocytes isolated from rat hypertrophied heart, Acta Physiologica Sinica, 1999, 51 (5): 588.
12. Bean, B. P., Two kinds of calcium channels in canine atrial cells: differences in kinetics, selectivity, and pharmacology, J. Gen. Physiol., 1986, 86: 1.
13. Bells, B., Melecot, C. D., Hescheler, J. et al., "Rundown" of the Ca^{2+} current during whole-cell: the role of phosphorylation and intracellular calcium, Pflugers Arch., 1988, 411: 352.
14. Barbara, K. T., Allen, I. C., Phillip, T. F., Structure of ionomycin ——a novel diacidic polyether antibiotic having high affinity for calcium ions, Journal of the American Chemical Society, 1979, 101(12): 3344.
15. Liu Anxi, Chen Shoutong, Ion channel in cell membranes (in Chinese), Beijing: Central National College Press, 1990.
16. Wu Dongmei, Lu Jiyuan, Wu Bowei, Class Ⅲ anti-arrhythmia drug E-4031 potentials Na^+/Ca^{2+} exchange in rat ventricular myocytes, Acta Pharmacol Sin, 2000, 21(3): 249.
17. Liu Gongxin, Yang Yingzhen, Na^+/Ca^{2+} exchange in ventricular myocytes, Chinese Journal of Cell Biology (in Chinese), 1998, 20(3): 129.
18. Cai Zhiwei, Ma Lian, Na^+-Ca^{2+} exchange and Na^+-Ca^{2+} exchanger in ventricular myocytes, Foreign Medical Sciences Section of Pathophysiology and Clinical Medical (in Chinese), 1998, 18 (1): 70.
19. Liang Yong, Wang Xiaoliang, Na^+/Ca^{2+} exchange current in ventricular myocytes, Progress in Physiological Sciences (in Chinese), 1999, 30 (2): 155.

(Received March 4, 2002)