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ION SELECTIVITY OF Ba^{2+} INWARD CURRENT OSCILLATIONS IN *RAS*-TRANSFORMED FIBROBLASTS THAT ELICIT CYTOPLASMIC Ca^{2+} OSCILLATIONS BY BRADYKININ

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SUMMARY: Ion selectivity of divalent cations on Ba^{2+} inward current oscillations was examined by voltage-clamp recording in *v*-Ki-*ras*-transformed NIH/3T3 (DT) fibroblasts where repetitive transient increases in cytoplasmic Ca^{2+} concentration were evoked by bradykinin. Application of bradykinin onto DT cells in 50 mM Ba^{2+} solution initiated Ba^{2+} inward current oscillations. The inward currents were inhibited in equimolar Sr^{2+} or Ca^{2+} solutions. Ba^{2+} current oscillations were dependent upon extracellular Ba^{2+} concentration. The results suggest that inward current oscillations are highly selective to Ba^{2+}.

Nonexcitable cells generate repetitive transient increases in intracellular free calcium concentration, designated as cytoplasmic Ca^{2+} oscillations, when stimulated with hormones or growth factors (1). Cytoplasmic Ca^{2+} oscillations require the increased Ca^{2+} entry across the plasma membrane to charge internal Ca^{2+} stores (2-4). Another divalent cation such as Mn^{2+} can enter through the Ca^{2+} entry pathway during cytoplasmic Ca^{2+} oscillations (3). It has been reported that cytoplasmic Ca^{2+} oscillations are induced by bradykinin or serum in *v*-Ki-*ras*-transformed NIH/3T3 (DT) cells, but not in control 3T3 fibroblast cells (5). By patch voltage-clamp measurement on *ras*-transformed DT cells, we have recorded a recurrent inward current (Ba^{2+} current oscillation) which is evoked by bradykinin that stimulates phosphoinositide metabolism (6). The Ba^{2+} inward current oscillations correspond in time with cytoplasmic Ca^{2+} oscillations and are modulated by intracellular cyclic AMP or phorbol esters (7), which also modify cytoplasmic Ca^{2+} oscillations to the same direction (8). This suggests that Ba^{2+} inward currents are closely related with

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cytoplasmic Ca^{2+} oscillations. In order to further characterize this divalent cation influx, we examined selectivity of divalent cations on Ba^{2+} inward current oscillations.

METHODS

Kirstein murine sarcoma virus-transformed NIH/3T3 (DT) cells (8) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum. Cells were soaked with Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} and were then detached by agitation. Cells (2 x 10^5) were reseeded onto polyornithine-coated dishes (35 mm in diameter), and kept for 2-6 days in the growth medium before use. To measure inward Ca^{2+} currents, cells were incubated at 35 °C in the Ba^{2+} solution (50 mM $BaCl_2$, 30 mM NaCl, 10 mM CsCl, 25 mM glucose, 25 mM tetraethylammonium chloride, 0.1 μM tetrodotoxin and 10 mM Hepes, pH 7.3) (9). Solution for filling patch pipettes contained 150 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA (buffered by 1 N NaOH), 0.4 mM sodium ATP and 10 mM Hepes, pH 7.3, adjusted with NaOH (9). To examine ion selectivity, 50 mM Ba^{2+} was replaced with 50 mM Sr^{2+} or Ca^{2+}. Choline chloride was used to replace Ba^{2+} for making various concentrations of Ba^{2+}. Cells were voltage-clamped by using patch-pipettes in "whole-cell" mode (10), and current was amplified by using single-electrode voltage-clamp amplifiers (Axoclamp-2A) in discontinuous mode, sampling at 3-8 kHz. Currents were filtered at 1 kHz and displayed on a Nihonkoden thermal array recorder.

RESULTS AND DISCUSSION

Whole cell Ca^{2+} currents were recorded by a patch electrode in a *ras*-transformed NIH/3T3 cell in perfusion medium containing either 50 mM Ba^{2+}, Sr^{2+} or Ca^{2+} as charge carriers at a holding membrane potential of about -40 mV. Stimulation with 100 nM bradykinin or 20 μM dibutyryl cyclic AMP or fetal calf serum elicited repetitive transient inward currents in more than 250 cells soaked only in 50 mM Ba^{2+} solution, as reported previously (7,8), but not in Sr^{2+} nor Ca^{2+} solutions tested in 30 cells. These recurrent inward currents in the presence of 50 mM Ba^{2+} correspond well in time with the repetitive increase in cytoplasmic Ca^{2+} concentration examined in separate cells. To assess whether Ba^{2+} influx acts to recharge Ca^{2+} pools during cytoplasmic Ca^{2+} oscillations, interaction between Ba^{2+} and other divalent cations was precisely examined.

Ion selectivity of inward current oscillations observed in 50 mM Ba^{2+} solution was also confirmed by replacing extracellular 50 mM Ba^{2+} with 50 mM Ca^{2+} or Sr^{2+}. Inward current oscillations suddenly ceased (Fig. 1A), and then suddenly recovered after Ca^{2+} was washed out (6 cells). Substitution of Ba^{2+} by equimolar Sr^{2+} decreased the inward current oscillation gradually, and finally stopped. This effect was also reversible (5 cells) (Fig. 1B).

To further characterize the difference in the inhibitory effect of external Ca^{2+} or Sr^{2+} on Ba^{2+} current oscillations, we measured the concentration of Ca^{2+} and Sr^{2+} required for suppression. The typical experiments performed in one DT cell

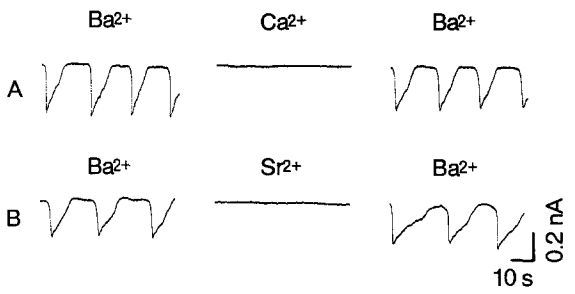

Fig. 1. **Ba^{2+} current oscillation in 50 mM Ba^{2+}, Ca^{2+} or Sr^{2+} solutions in a DT cell.** The cell was voltage-clamped at -31 mV. Ba^{2+} current oscillations evoked by 10% fetal calf serum were observed in 50 mM Ba^{2+} solution. The current oscillation stopped after switching to 50 mM Ca^{2+} solution (A) or 50 mM Sr^{2+} solution (B). Current oscillations were regained after superfusion with 50 mM Ba^{2+} solution.

are shown in Figs. 2 and 3. In the experiment in Fig. 2, the DT cell was first superfused with 50 mM Ba^{2+} solution where current oscillations were observed (Inset). Increasing Ca^{2+} from 0.2 to 0.5-2 mM terminated the current oscillations. The mean concentration required for 50% inhibition of Ba^{2+} current oscillations (IC_{50}) by Ca^{2+} was 0.53 ± 0.6 mM (mean S.E.M., n=6).

On the other hand, amplitude and frequency of current oscillation were unchanged even at 15 mM Sr^{2+} plus 35 mM Ba^{2+}. Increasing Sr^{2+} from 15 mM to 17-20 mM resulted in a complete inhibition of the oscillations in the same DT cell (inset and plot of Fig. 3). IC_{50} of Sr^{2+} for Ba^{2+} current oscillation was 15 ± 1 mM (n=3).

Extracellular Ba^{2+} was essential for current oscillations, since oscillations were terminated when extracellular 50 mM Ba^{2+} was replaced with 75 mM Na^{+} or choline (data not shown). The dependence of Ba^{2+} inward current oscillations on external Ba^{2+} was determined by measuring the effect of gradual decrease in external Ba^{2+} on the amplitude and frequency of Ba^{2+} current oscillations. As shown in Fig. 4A, the cell first showed current oscillations at 50 mM Ba^{2+}. Decreasing external Ba^{2+} from 50 to 6-8 mM resulted in decrease in amplitude with constant frequency (Fig. 4A and B). Further decreases in external Ba^{2+} suppressed the oscillations, which resumed after restoring to 10 mM Ba^{2+} or more. EC_{50} of external Ba^{2+} for Ba^{2+} current oscillations was calculated to be 6.7 ± 1.2 mM (n=8).

The data presented here show that oscillatory inward currents are highly responsive to Ba^{2+} in DT cells. External Ca^{2+} acted as an inhibitor on Ba^{2+} current oscillations rather than as a permeable ion, though the concentration required was higher than Ni^{2+} (about 50 μM for Ni^{2+}) (6). The blocking effect of Sr^{2+} was much weaker than that of Ca^{2+}. Divalent cation influx through high threshold voltage-activated Ca^{2+} channels, L-type channels, can be usually recorded in either Ba^{2+}, Sr^{2+} or Ca^{2+} solutions in various types of cell (11,12), including control Swiss/3T3 cells (13) or *ras*-transformed NIH/3T3 cells (14). Therefore, Ba^{2+}

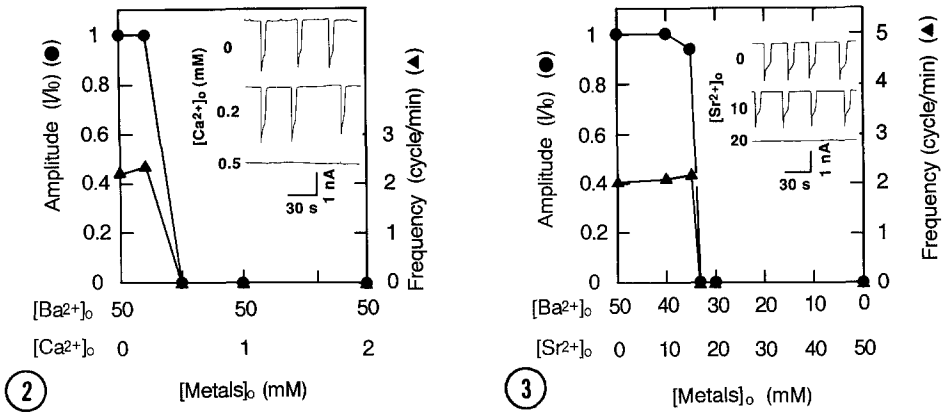

Fig. 2. **Inhibition of Ba^{2+} inward current oscillation by Ca^{2+}.** A DT cell prestimulated with 100 nM bradykinin in 50 mM Ba^{2+} solution produced Ba^{2+} current oscillations at a holding potential of -38 mV. Then the cell was exposed to 50 mM Ba^{2+} solution supplemented with 0.2, 0.5, 1 and 2 mM Ca^{2+}. Current traces recorded at 0, 0.2 and 0.5 mM Ca^{2+} are shown in inset. Amplitude of currents in 50 mM Ba^{2+} solution plus various concentrations of Ca^{2+} (I) is normalized to that in 50 mM Ba^{2+} solution (I_0) (●). Frequency of the current oscillation against Ca^{2+} concentration is plotted (▲).

Fig. 3. **Oscillatory inward currents in various combination of Ba^{2+} and Sr^{2+} mixture.** Inward current oscillations in the cell in Fig. 2 were observed when it was soaked in the standard 50 mM Ba^{2+} solution. External 50 mM solution was then replaced with different ratios of Ba^{2+} and Sr^{2+} concentrations (mM): 50/0; 40/10; 35/15; 33/17; 30/20 and 0/50. Current traces obtained at the ratio (mM) of Ba^{2+} and Sr^{2+} with 50/0, 40/10 and 30/20 are shown in inset. Normalized amplitude (I/I_0) (●) and frequency (▲) of the currents are plotted as a function of Sr^{2+} and Ba^{2+} concentrations. I, amplitude of currents at various concentrations of Sr^{2+}; I_0, one at 0 mM Sr^{2+} and 50 mM Ba^{2+}.

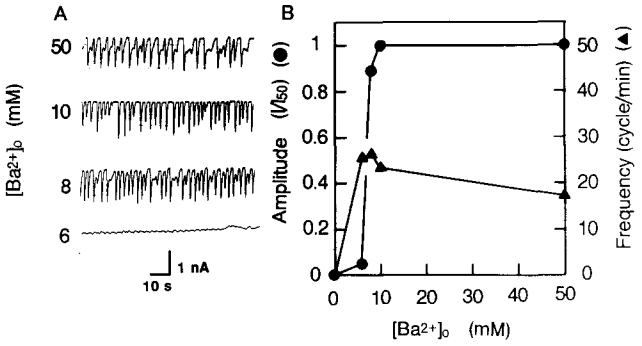

Fig. 4. **Dependency of Ba^{2+} current oscillations on extracellular Ba^{2+} concentration.** Inward current oscillations were set up by 100 nM bradykinin in a DT cell soaked in the standard 50 mM Ba^{2+} solution. The solution was then replaced by solutions with various Ba^{2+} concentrations (6, 8 and 10 mM). A. Current traces recorded at 50, 10, 8 and 6 mM Ba^{2+} solutions. B. Plots of normalized amplitude (●) and frequency (▲) of the currents in A. I, amplitude of currents in various Ba^{2+} concentrations; I_{50}, one in 50 mM Ba^{2+} solution.

selectivity of these inward oscillatory currents differs from that for voltage-gated Ca^{2+} currents, suggesting that Ba^{2+} oscillatory currents are generated by permeation through unidentified divalent cation channels or a modified state of known ones. Because Ba^{2+} is not adequate for measurement of repetitive changes in cytoplasmic Ca^{2+} concentration, it is rather difficult to show whether these Ba^{2+} entry pathways are directly related with cytoplasmic Ca^{2+} oscillations. However, the fact that a receptor-mediated Ca^{2+} entry blocker, SK&F96365 inhibits both Ba^{2+} current oscillations and cytoplasmic Ca^{2+} oscillations suggests that Ba^{2+} may enter during cytoplasmic Ca^{2+} oscillations (5,6).

In conclusion, Ba^{2+} current oscillations are specific to external Ba^{2+} and affected by external Ca^{2+} or Sr^{2+}. Since Ba^{2+} current oscillations were observed mostly in DT cells but not in control NIH/3T3 cells, they are unique responses acquired after *ras*-transformation of the fibroblast cells. A further validation is necessary and will be undertaken by measuring Ca^{2+} concentrations with fura-2 image and Ca^{2+} currents simultaneously, even though recordable Ca^{2+} currents might be much smaller than Ba^{2+} currents.

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