A slowly inactivating calcium current works as a calcium sensor in calcitonin-secreting cells

H. Scherübl, G. Schultz and J. Hescheler

Pharmakologisches Institut der Freien Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, FRG

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Calcitonin (CT)-secreting cells (C-cells) are remarkably sensitive to changes in the extracellular Ca^{2+} concentration. In order to detect the mechanism by which C-cells monitor Ca^{2+}, we compared a C-cell line responding to Ca^{2+} (rMTC cells) with another one known to have a defect in this Ca^{2+} signal transduction (TT cells). Rises of the Ca^{2+} concentration caused rMTC cells to depolarize and/or elicited spontaneous action potentials. Under voltage-clamp conditions, rMTC cells showed a slowly decaying Ca^{2+} inward current which was sensitive to dihydropyridines but not to Ni^{2+} at a low concentration. In contrast, the 'defective' TT cells neither depolarized nor fired action potentials with high Ca^{2+}; they only exhibited an Ni^{2+}-sensitive, transient Ca^{2+} current. The data strongly suggest that the slowly inactivating Ca^{2+} current is a prerequisite for Ca^{2+}-sensitivity of C-cells and that fast inactivating channels are not sufficient to act as sensors of the extracellular Ca^{2+} concentration.

Calcitonin-secreting cell; rMTC cell; TT cell; Calcium current

1. INTRODUCTION

Calcitonin (CT)-secreting cells (C-cells) are preferentially located in the thyroid gland and are considered to play an important role in the complex regulatory network of Ca^{2+} homeostasis. Previous reports on the permanent C-cell line rMTC [1,2] revealed a close correlation between the extracellular Ca^{2+} concentration and both the intracellular Ca^{2+} concentration [3] and CT release. The Ca^{2+}-dependent CT release was blocked by nitrendipine [4] or verapamil [5], mimicked by Bay K 8644 [4,6] and potentiated by (+)202–791 [7]. In contrast, C-cells of the TT-line [8], due to an unknown defect, are unable to respond to Ca^{2+}, but CT secretion rises after electropermeabilization [9]. Although these findings have suggested a prominent role of voltage-dependent Ca^{2+} channels [4,7,10] for the Ca^{2+} sensitivity of C-cells, no electrophysiological data supporting this hypothesis are yet available. Here we report on whole cell recordings of voltage-dependent Ca^{2+} currents in rMTC and TT cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Rat MTC cells (rMTC 44-2 C-cell line) were grown in monolayer culture using DMEM (Biochrom, Berlin, FRG) supplemented with 15% horse serum and 2.5% fetal calf serum (Gibco, Paisley, UK). Human MTC cells (TT cells, hMTC C-cell line) were grown in the same way except for the use of RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 16% fetal calf serum.

Correspondence address: J. Hescheler, Pharmakologisches Institut der Freien Universität Berlin, Thielallee 69–73, D-1000 Berlin 33, FRG

2.2. *Secretion experiments*

Confluent rat and human C-cells were preincubated with serum-free DMEM or RPMI medium for 2 h, washed twice with PBS buffer and incubated again with medium containing test agents or vehicle for 1 h. Then medium was removed and stored at −20°C until assayed for immumoreactive CT [12]. CT secretion was standardized to cellular protein content.

2.3. *Electrophysiology*

For electrophysiological investigations, cells were cultured on small glass slides (density 2–5 cells/mm^2). After transfer into a chamber (0.2 ml), the attached cells were superfused at a constant rate of about 5 ml/min. The whole cell membrane currents were measured according to the method described by Hamill et al. [11] for special modifications (see [13]). The patch electrodes had an average resistance of 5 MΩ (open diameter about 1 μm), which allowed to obtain GΩ seals within about 30 s. After disruption of the membrane patch under the tip of the patch pipette, a whole cell configuration was obtained, suitable for measuring membrane currents under voltage-clamp conditions (see [11]). Or, alternatively and in order to avoid a major disturbance of the cytoplasma, e.g. change of the intracellular Ca^{2+} buffering capacity, we assessed the cytoplasma using the nystatin method [14,15].

2.4. *Solutions*

External solution E1 contained (in mM): 135 NaCl, 1.2 $CaCl_2$, 1 $MgCl_2$, 5.4 KCl, 10 glucose and 10 Hepes (pH 7.4 with NaOH, 37°C). Solutions E2–E4 contained: 135 or 125 TEA-Cl, 1 $MgCl_2$, 10 glucose, 10 Hepes (pH 7.4 with TEA-OH, 37°C), TTX (200 nM) and 1.2 $CaCl_2$ (solution E2) or 10.8 $CaCl_2$ (solution E3) or 10.8 $BaCl_2$ (solution E4). Solutions E5–E6 contained: 135 or 125 NaCl, 1 $MgCl_2$, 5.4 CsCl, 10 glucose, 10 Hepes (pH 7.4 with NaOH, 37°C), 200 nM TTX and 1.2 $BaCl_2$ (solution E5) or 10.8 $BaCl_2$ (solution E6). Pipette solution I1 contained (in mM): 90 K-aspartate, 50 KCl, 4 $MgCl_2$, 10 Hepes (pH 7.4 with KOH, 37°C), 3 Na_2-ATP and was supplemented with freshly prepared nystatin (100 200 μg/ml). I2 contained: 120 CsOH, 120 L-aspartate, 20 CsCl, 4 $MgCl_2$, 3 Na_2-ATP, 10 Hepes (pH 7.4 with TEA-OH) and was supplemented with freshly prepared nystatin (100 200 μg/ml). I3 contained: 100 CsCl, 40 CsOH, 4 $MgCl_2$, 3 Na_2-ATP, 10 Hepes (pH 7.4 with CsOH, 37°C), 10 EGTA, 6.0 $CaCl_2$; the calculated free concentration of Ca^{2+} in this solution was 0.1 μM.

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2.5. *Statistics*

Data are presented as the mean ± SE. Statistical significance was assessed by the Wilcoxon rank sum test.

3. RESULTS

3.1. *Secretion experiments*

Table I shows the effects of high Ca^{2+} and the Ca^{2+} channel agonist Bay K 8644 on CT release from rMTC and TT cells. Rising Ca^{2+} from 1.1 to 2.0 mM or adding 10 μM Bay K 8644 increased CT secretion from rMTC cells but did not affect CT release from TT cells, agreeing with previous reports [6,9].

3.2. *Spontaneous action potentials*

rMTC cells exhibited resting potentials of −44.5 ± 1.6 mV ($n=38$). Rising the Ca^{2+} concentration from 1.2 to 1.8 mM elicited spontaneous action potentials (Fig. 1 1A) and/or depolarized the cells by 12.1 ± 2.3 mV ($n=9$) . The action potentials evoked by high Ca^{2+} could be reversibly suppressed by the Ca^{2+} channel blocker isradipine (PN 200-110, 1 mM) (Fig. 1C). At 1.2 mM Ca^{2+}, 1 mM Bay K 8644 often induced a few initial spikes but then led to a continuous depolarisation by 16.3 ± 2.9 mV ($n=10$). Washing out Bay K 8644 repolarized the cells (Fig. 1E).In contrast, the resting membrane potential of TT cells was affected neither by high Ca^{2+} (Fig. 1B) nor isradipine (Fig. 1D) nor Bay K 8644 (Fig. 1F); their resting potential stayed at −37.9 ± 1.8 mV ($n=13$).

3.3. *Ca^{2+} currents during voltage-clamp steps*

To measure the Ca^{2+} currents, K^+ and Na^+ currents were blocked with Cs^+, TEA and tetrodotoxin. Under these conditions and with 1.2 mM Ca^{2+} as divalent charge carrier, rMTC cells exhibited slowly inactivating inward currents (Fig. 2A) which had a maximal current density of 9.3 ± 0.7 pA/pF ($n=12$) at −10 mV. The current-voltage (*IV*) relationship revealed a threshold at about −50 mV and an apparent reversal potential at 30 mV. Increasing the Ca^{2+} concentration to 10.8 mM raised the maximal current 2.5-fold and shifted the threshold of the *IV*-curve to about −40 mV (data not shown). Semilogarithmic plotting of the Ca^{2+} current-inactivation during depolarisation pulses for 3 s [16] revealed two inactivation constants τ_1 and τ_2 in rMTC cells. With 1.2 mM Ca^{2+} and at 37°C, τ_1 amounted to 168 ± 11 ms and τ_2 to 4.9 ± 0.7 s ($n=5$) for depolarisation steps from −80 to −10 mV.

Under 1.2 mM Ca^{2+}, TT cells showed negligibly small currents. At 10.8 mM Ca^{2+}, transient inward currents were detectable which completely inactivated within about 40 ms (Fig. 2B). Compared to the current of rMTC cells, the current density was about 4.5-fold smaller and the *IV* curve was obviously shifted to the left. In agreement with the described properties of the

Table I

Effects of Ca^{2+} and Bay K 8644 on calcitonin secretion.

	Calcitonin release	
	rMTC cells ($pg \cdot mg\ protein^{-1} \cdot h^{-1}$)	TT cells ($ng \cdot mg\ protein^{-1} \cdot h^{-1}$)
Control	126 ± 5.8	4.58 ± 0.32
10 μM Bay K 8644	229 ± 8.0*	4.70 ± 0.27^{ns}
2 mM Ca^{2+}	257 ± 10.2*	4.37 ± 0.24^{ns}

Basal Ca^{2+} concentration was 1.1 mM. Mean ± SEM ($n=5$).
*$P<0.01$; ^{ns}not significant to control

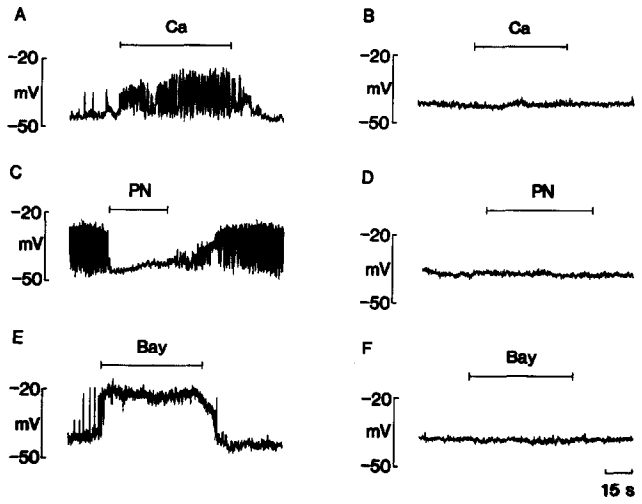

Fig. 1. Effects of Ca^{2+}, a Ca^{2+} channel blocker and an agonist on membrane potentials in C-cells. The effects of 1.8 μM Ca^{2+} (Ca) (A and B), 1 μM isradipine (PN) (C and D) and 1 μM Bay K 8644 (Bay) (E and F) on membrane potentials of rMTC (left) and TT (right) cells are shown. The substances were added as indicated by the horizontal lines. Solutions: pipette solution I1 and external solution E1. External solution E1 with 1.8 instead of 1.2 μM Ca^{2+} was applied as marked in (A) and (B) and throughout in (C) and (D).

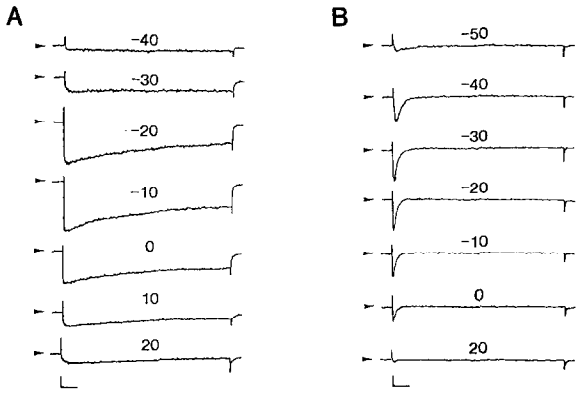

Fig. 2. Whole-cell recordings of Ca^{2+} currents in C-cells. Original current traces of rMTC (A) and TT (B) cells are shown during 300 ms long voltage clamp pulses from −80 mV to various test potentials as indicated by the numbers. The membrane patch under the tip of the pipette was disrupted and free access to the cytoplasm was obtained. Solutions: pipette solution I3 for both rMTC and TT cells; external solutions E2 (1.2 μM Ca^{2+}) for rMTC and E3 (10.8 μM Ca^{2+}) for TT cells. Vertical and horizontal calibration marks correspond to 30 pA and 30 ms. Cell capacity: 12.1 pF (A) and 15.9 pF (B).

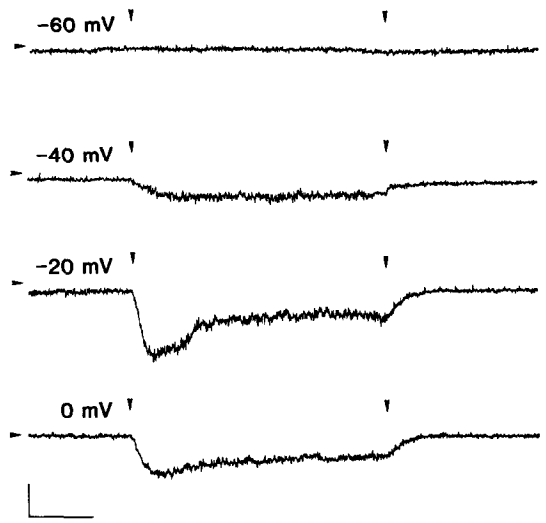

Fig. 3. Effect of Ba^{2+} on inward currents in a single rMTC cell at various holding potentials. The rMTC cell was voltage-clamped at the indicated potentials for 2 min. For the period marked in between the vertical arrows Ba^{2+} was raised from 1.2 mM (solution E5) to 10.8 mM (solution E6); pipette solution I2. Horizontal arrows mark the zero current level. Calibration marks correspond to 15 s and 20 pA. Cell capacity: 14.3 pF.

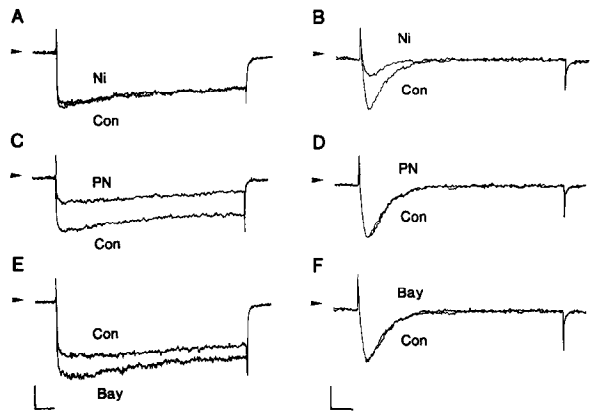

Fig. 4. Effects of Ca^{2+} channel blockers and an agonist on Ca^{2+} currents in C-cells. The effects of 10 μM Ni^{2+} (Ni) (A and B), 1 μM isradipine (PN) (C and D) and 1 μM Bay K 8644 (Bay) (E and F) on currents of rMTC (left) and TT (right) cells are shown. CON refers to the controls. Test pulses from -60 to 0 mV and from -80 to -30 mV were applied to rMTC and to TT cells, respectively. Solutions: Pipette solution I3 and external solution E4. Arrows mark the zero current level. In (A, C and E), calibration marks correspond to 20 ms and 100 pA, in (B, D and F) to 10 ms and 30 pA.

transient T-type Ca^{2+} currents [17,18], the threshold was about -60 mV and the maximum (5.1 $\pm$ 0.5 pA/pF, $n=9$) occurred at about -30 mV.

The permeability ratio of Ba^{2+}/Ca^{2+} (10.8 mM) amounted to 2.1 $\pm$ 0.2:1 ($n=5$) for Ca^{2+} channels in rMTC cells and to 1.1 $\pm$ 0.1:1 ($n=4$) for Ca^{2+} channels in TT cells. The midpoint voltage of the steady-state inactivation curve of the Ca^{2+} current as measured with 10.8 mM Ca^{2+} was -24 $\pm$ 2.5 mV ($n=5$) for rMTC cells and -59 $\pm$ 2.9 mV ($n=4$) for TT cells.

3.4. *Effect of Ba^{2+} on inward currents*

To provide direct evidence for a steady-state conductivity of Ca^{2+} channels underlying the Ca^{2+} sensitivity of rMTC cells, we voltage-clamped C-cells at fixed holding potentials for several minutes and measured the current response to rises of Ba^{2+} from 1.2 to 10.8 mM. At -40 mV, rMTC cells produced an inward current which was maximal (23.0 $\pm$ 8.6 pA, $n=9$) after about 5 s and then slowly decayed. Fig. 3 3 demonstrates the voltage dependence of this inward current for potentials between -60 to 0 mV. The elicited Ba^{2+} inward current displayed a U-shaped voltage dependence with a threshold of about -50 mV. In contrast to rMTC cells, TT cells failed to display any inward current in analoguous experiments (data not shown).

3.5. *Pharmacological characterization*

The difference between rMTC and TT cells with respect to their Ca^{2+} currents became more apparent by their different pharmacology. Ni^{2+} at low concentrations is known to specifically block T-type currents [17]. In line with these reports, the fast inactivating Ca^{2+} current of TT cells was inhibited by 65 $\pm$ 3% ($n=5$) under 10 μM Ni^{2+} (Fig. 4B); but the slowly inactivating current of rMTC cells was not or only minimally affected (Fig. 4A). A reverse sensitivity was found in the case of dihydropyridines (Fig. 4C-F). Isradipine (PN 200-110, 1 μM) suppressed the Ca^{2+} current of rMTC cells by 57 $\pm$ 5% ($n=6$) and the Ca^{2+} channel agonist Bay K 8644 (1 μM) stimulated it by 34 $\pm$ 4% ($n=4$). Both isradipine and Bay K 8644 did not affect the current of TT cells ($n=7$).

4. DISCUSSION

A major role of dihydropyridine-sensitive Ca^{2+} currents for the Ca^{2+} sensitivity of C-cells has been suggested by the effects of Ca^{2+}, organic Ca^{2+} channel blockers and agonists on calcitonin secretion and cytosolic Ca^{2+} concentration in C-cells. (i) The Ca^{2+}-induced calcitonin release is blocked by Ca^{2+} channel blockers [4,5,10]. (ii) The calcitonin release is stimulated by Ca^{2+} channel openers [4,6,10,12]. (iii) The intracellular Ca^{2+} is highly dependent on the extracellular Ca^{2+} concentration [3]. (iv) The intracellular Ca^{2+} rises with Bay K 8644 and falls with nifedipine [6]. (v) Increasing the extracellular K^+ concentration causes a depolarisation, an increase in the cytosolic Ca^{2+} and a subsequent calcitonin release [1,3,19].

Our electrophysiological studies provide direct evidence for a voltage-dependent, dihydropyridine-sensitive, long lasting Ca^{2+} current in rMTC-cells and its essential role in the Ca^{2+} sensitivity of C-cells as

evidenced by C-cells of the 'defective' TT cell line which lack this Ca^{2+} current and are unable to regulate calcitonin secretion in response to Ca^{2+}; the fast inactivating Ca^{2+} current that can be detected in TT cells fails to substitute as sensor of the extracellular Ca^{2+} concentration. The differing Ca^{2+} sensitivity of rMTC and TT cells cannot be attributed to species differences (rat and human, respectively), as normal human C-cells and several primary cultures of human C-cell carcinoma have been shown to respond to Ca^{2+} [10,20,21].

To monitor the extracellular Ca^{2+} concentration, C-cells need to have a steady state conductivity for Ca^{2+}. Voltage-clamping rMTC cells near their resting potential demonstrated a steady state-inward current through Ca^{2+} channels which depended on the concentration of the divalent charge carrier. Analyzing the Ca^{2+} current-inactivation we determined a fast (168 ms) and a slow inactivation time constant of 4.9 s. With physiological Ca^{2+} concentrations and at 37°C, inactivation constants as long as 4.9 s have not been reported for other cells [22], e.g. in cardiocytes, inactivation occurs within about 100 ms [23]. Whether the two inactivation time constants are due to different inactivated states of the Ca^{2+} channel [24] or the presence of different types of Ca^{2+} channels remains to be evaluated.

C-cells are known to generate tetrodotoxin- and D600-sensitive action potentials [19,25]. Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels apparently plays an important role therein. Increasing Ca^{2+} or adding Bay K 8644 induced action potentials and/or depolarized rMTC cells; isradipine suppressed the Ca^{2+}-evoked action potentials. In addition, the similarity between the activation threshold of the Ca^{2+} current and the resting potential of rMTC cells argues for a role of the slowly inactivating Ca^{2+} current in the generation of spontaneous activity (compare with [26]). T-type Ca^{2+} currents regarded to be involved in spontaneous activity in other cell types [27,28] were not seen in rMTC cells. Moreover, TT cells which exhibited only fast inactivating Ca^{2+} currents did not display spontaneous action potentials. Thus T-type Ca^{2+} currents by themselves seem not to be sufficient to generate pacemaking activity.

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