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Properties of a new calcium-permeable single channel from tracheal microsomes

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Abstract

After the incorporation of the tracheal microsomal membrane into bilayer lipid membrane (BLM), a new single channel permeable for calcium was observed. Using the BLM conditions, 53 mM Ca^{2+} in *trans* solution versus 200 nM Ca^{2+} in *cis* solution, the single calcium channel current at 0 mV was 1.4–2.1 pA and conductance was 62–75 pS. The channel Ca^{2+}/K^{+} permeability ratio was 4.8. The open probability (*P*-open) was in the range of 0.7–0.97. The *P*-open, measured at −10 mV to +30 mV (*trans-cis*), was not voltage dependent. The channel was neither inhibited by 10–20 μM ruthenium red, a specific blocker of ryanodine calcium release channel, nor by 10–50 μM heparin, a specific blocker of IP_3 receptor calcium release channel, and its activity was not influenced by addition of 0.1 mM MgATP. We suggest that the observed new channel is permeable for calcium, and it is neither identical with the known type 1 or 2 ryanodine calcium release channel, nor type 1 or 2 IP_3 receptor calcium release channel.

Keywords: Trachea; Microsome; Single channel; Calcium channel; Lipid bilayer

1. Introduction

Regulation of intracellular calcium concentration in tracheal smooth muscle cells plays an important role in many physiological processes. After agonist stimulation of tracheal cells, intracellular calcium increases due to Ca^{2+} influx and Ca^{2+} release from endoplasmic reticulum. The Ca^{2+} release from the endoplasmic reticulum in smooth muscle cells is mediated through both IP_3R and RYR channels [1–6]. IP_3 was shown to induce a monotonic increase in Ca^{2+} in permeabilized canine tracheal smooth muscle cells that could be blocked by heparin [6]. Intracellular calcium oscillations in porcine tracheal smooth muscle cells involving repetitive Ca^{2+} release and reuptake from RYR channels were reported [1], and it was recently documented that ryanodine and caffeine increased the incidence of Ca^{2+} sparks, indicating that RYR receptor was present in the tracheal cells [6].

However, single channel properties of calcium channels from tracheal microsomes have not been studied so far. Therefore, in the present work we looked for calcium channels in tracheal microsomal membranes incorporated into bilayer lipid membranes (BLM).

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor calcium channel; RYR, ryanodine receptor calcium channel; 8-pCPT-cGMP, 8-(4-*para*-chlorophenylthio)guanosine-3′,5′-monophosphate; Ca^{2+}, free Ca^{2+} concentration; BLM, bilayer lipid membrane

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2. Materials and methods

Chemicals were from Sigma, and lipids were from Avanti Polar Lipids. Tracheal membranes were prepared by the following procedure at 4°C. Bovine tracheal smooth muscle was isolated and homogenized in buffer 1 (8% (w/v) sucrose, 20 mM MOPS, pH 7.4) containing protease inhibitors (PMSF 0.2 mM, leupeptin 0.2 μg/ml, benzamidine 1 mM, calpain I inhibitor 1 μg/ml). The homogenate was filtered through cheesecloth and the filtrate centrifuged ($10\,000\times g$, 10 min). The supernatant was ultracentrifuged ($130\,000\times g$, 60 min) and the resulting pellet was resuspended in buffer 1. This suspension was layered on a discontinuous sucrose gradient (8%, 30%, 40% sucrose) followed by ultracentrifugation in an SW28 rotor ($160\,000\times g$, 60 min). The 8%/30% sucrose interface of the gradient was frozen in aliquots at −80°C and later used for BLM study.

BLM were formed across a hole (diameter 0.1–0.2 mm) separating the *cis* and *trans* chambers, using a mixture of dioleoylglycerophosphoethanolamine, dioleoylglycerophosphatidylserine and dioleoylglycerophosphatidylcholine at a molar ratio of 3:2:1 in *n*-decane (20 mg/ml), similarly as in previous studies [7,8]. During single channel measurements, the *trans* chamber contained 53 mM $Ca(OH)_2$, 50 mM KCl, buffered with HEPES (approx. 250 mM) to pH 7.4, and the *cis* chamber contained 250 mM HEPES, 50 mM KCl, and 1 mM EGTA buffered with Tris (approx. 113 mM) to pH 7.4. Ca^{2+}-free concentration in the *cis* chamber was 200 nM, calculated according to Schoenmakers et al. [9], and was adjusted using $CaCl_2$. In order to evaluate the Ca^{2+}/K^+ permeability ratio of the channel, we used a *trans* solution of 55 mM $Ca(OH)_2$ buffered with HEPES to pH 7.4, and a *cis* solution of 110 mM KOH buffered with HEPES to pH 7.4 containing 200 nM Ca^{2+}-free. The Ca^{2+}/K^+ permeability ratio (P_{Ca2+}/P_{K+}) was calculated according to Eq. 1 [10,11].

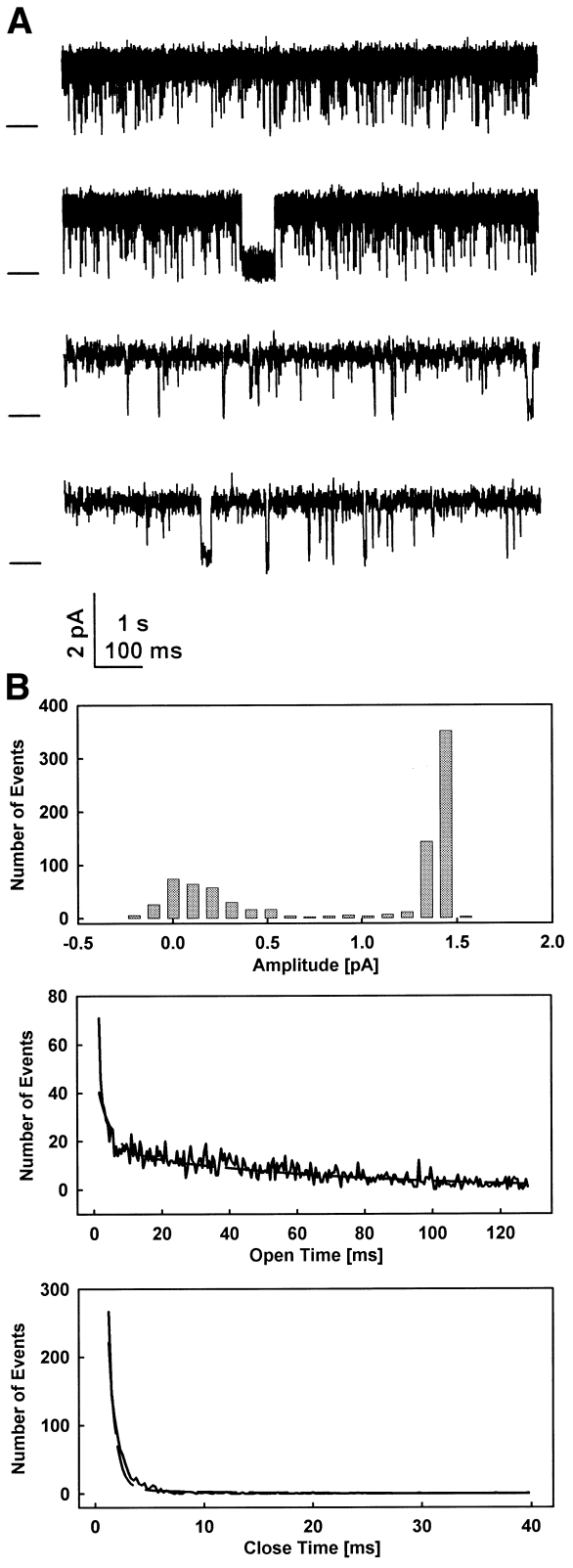
$$P_{Ca2+}/P_{K+} = [K^+]/4[Ca^{2+}]\times\exp(E_{rev}F/RT)\{\exp(E_{rev}F/RT)+1\} \quad (1)$$

The value of RT/F used was 25.2 mV at 20°C. E_{rev} is a reversal potential.

Tracheal microsomes, added to the *cis* chamber, were fused in the presence of 800 mM KCl and protease inhibitor leupeptin (5 μM). After the fusion of the microsomal membranes into BLM, non-fused microsomes and 800 mM KCl were removed by perfusion. Voltage was applied to the *trans* side of the chamber and the *cis* side was virtually grounded. Single channel current was measured by Axoclamp 1C (Axon Instruments, Foster City, CA). The data were acquired using Labmaster converter and acquisition software (Pclamp5, Axon Instruments). The single channel data were filtered at 1 kHz, digitized at 4 kHz and stored on an IBM-compatible 486 computer disk for later analysis. The Pclamp5 and Pclamp6 program was used to evaluate the data [7,8]. Open probability of channels, and the lifetimes of open and closed events were identified by 50% threshold analysis. The baseline level was obtained after leak current subtraction. The amplitude histogram was constructed from 2 min records of steady state recordings using the pSTAT program (Axon Instruments).

3. Results

After the incorporation of tracheal microsomal membranes into BLM using 800 mM KCl at the *cis* side, potassium channels were often seen (data not shown). Since we were interested in microsomal channels permeable for Ca^{2+}, the *cis* solution was perfused with a solution containing 50 mM KCl, 200 nM Ca^{2+}-free, buffered with HEPES-Tris to pH 7.4. After the perfusion, channels were observed in about 20% of the successful fusions of the vesicles into BLM. The channels passed calcium from the *trans* side to the *cis* side (Fig. 1A). In few experiments ($n = 3/14$), channels were seen which had properties of RYR or IP_3R calcium release channels. Since they were seen very seldom, we did not study them further. The frequently observed channels had very high open probability *P*-open = 0.7–0.97 ($n = 11/14$), and they were active at 200 nM Ca^{2+} at the *cis* side without addition of any activator. Examples of single calcium channel traces at low and high resolutions are shown in Fig. 1A. The channels were mostly open with short closing intervals. Amplitude histogram, open time and closed time histograms are shown in Fig. 1B. The single calcium channel current did not show regular substates and the open state



current at 0 mV was 1.4–2.1 pA ($n=11$). The data were fitted by two exponential times. The mean open time of the channel varied: $\tau_1 = 2.7 \pm 1.9$ ms and $\tau_2 = 51 \pm 25$ ms ($n=6$, ± S.D.).

Single channel traces obtained at different voltages are shown in Fig. 2A. *P*-open, in the range of −10 mV to +30 mV, measured during 5 s intervals, did not depend on voltage ($n=4$) (Fig. 2B). The single channel conductance, determined from the current-voltage relation in the range of −20 to +30 mV was 62–75 pS ($n=4$).

In order to evaluate the Ca^{2+}/K^+ permeability ratio of the channel, we used 55 mM $Ca(OH)_2$ buffered with HEPES to pH 7.4 as the *trans* solution, and 110 mM KOH buffered with HEPES to pH 7.4 containing 200 nM Ca^{2+}-free as the *cis* solution. The voltage dependence of the single channel current and the current-voltage relation are shown in Fig. 3. In these experimental conditions, the channel current increased at positive voltages (*trans-cis*) and decreased at negative ones (Fig. 3A). This suggests that the channel was permeable for Ca^{2+} over K^+. From the reversal potential of −24.6 mV, the Ca^{2+}/K^+ permeability ratio calculated according to Eq. 1 was 4.8 (Fig. 3B).

It was reported that tracheal cells have RYR and IP_3R intracellular calcium release channels [1–6,12]. Therefore, we tested specific inhibitors of the channels, ruthenium red and heparin. The open probability of the channel was not inhibited by 10–20 μM ruthenium red ($n=4/4$), a specific blocker of RYR calcium channel (Fig. 4). Ruthenium red did not influence open probability or mean open time of the channel; the changes were within the range of the standard deviation of the control values (Fig. 4B).

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Fig. 1. (A) The single calcium channel currents of microsomal membrane incorporated in BLM at low (top two traces) and high (bottom two traces) resolutions. The line on the left indicates the closed state of the channels. Filter 500 Hz. *Trans* solution: 53 mM $Ca(OH)_2$, 50 mM KCl, HEPES (approx. 250 mM), pH 7.4; *cis* solution: 250 mM HEPES, 50 mM KCl, 200 nM Ca^{2+}, Tris (approx. 113 mM), pH 7.4. (B) Amplitude histogram ($n=1100$ events), open ($n=3200$ events) and closed time histograms ($n=2200$ events) of the single calcium channel current of microsomal membrane incorporated in BLM. The data were fitted by two exponential times. Mean open times: $\tau_1 = 3.9$ ms and $\tau_2 = 64$ ms. Mean closed times: $\tau_1 = 0.6$ ms and $\tau_2 = 10$ ms.

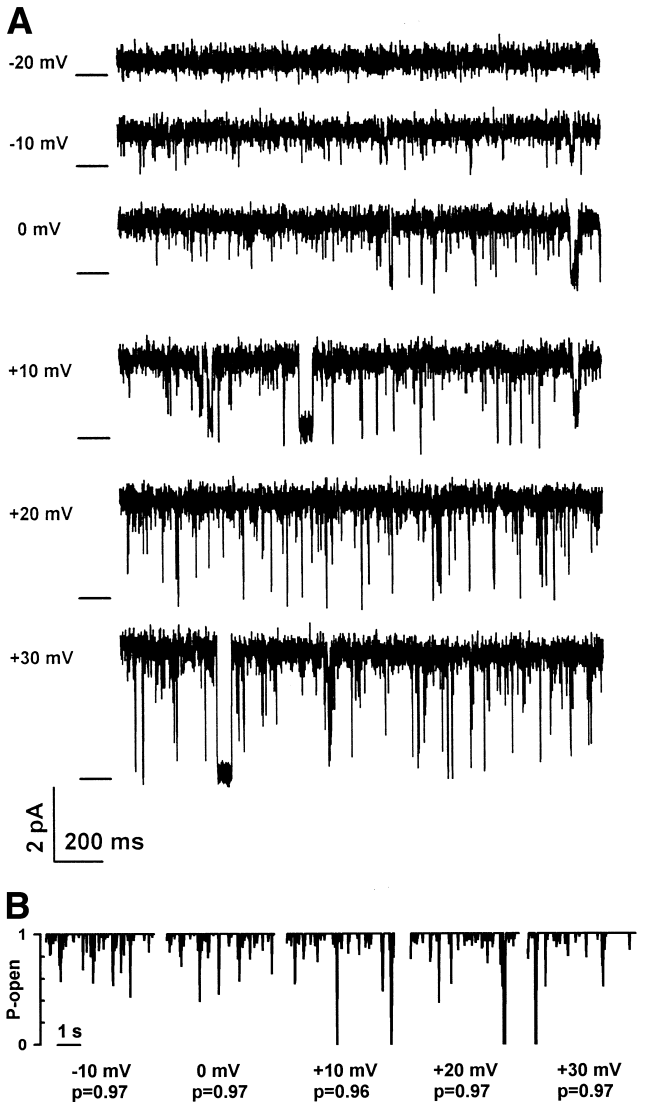


Fig. 2. (A) Voltage dependence of the single calcium channel current of microsomal membrane incorporated in BLM. Voltage is defined as *trans-cis*. Filter 500 Hz. The line on the left indicates the closed state of the channels. Same solutions as in Fig. 1. (B) Open probability of the single calcium channel currents of microsomal membrane incorporated in BLM at different voltages.

The specific blocker of IP_3R, heparin (10–50 μM), did not influence the calcium channel *P*-open and mean open time ($n = 5/5$); the changes were within the range of the standard deviation of the control values (Fig. 5). We tested 0.1 mM ATP, which is an activator of ryanodine and IP_3R calcium channel, and the compound did not change the open probability of the channels ($n = 4/4$) (data not shown). Similarly, the activator of cGMP-dependent protein kinase, 8-pCPT-cGMP (3 μM), did not change the channel open probability ($n = 3/3$) (data not shown).

4. Discussion

In the present work, we could demonstrate that microsomal membranes isolated from bovine trachea contain a channel passing calcium. The calcium channel had unusually high, voltage independent, open probability (*P*-open = 0.7–0.97) in the absence of any specific activators. Such high open probabil-

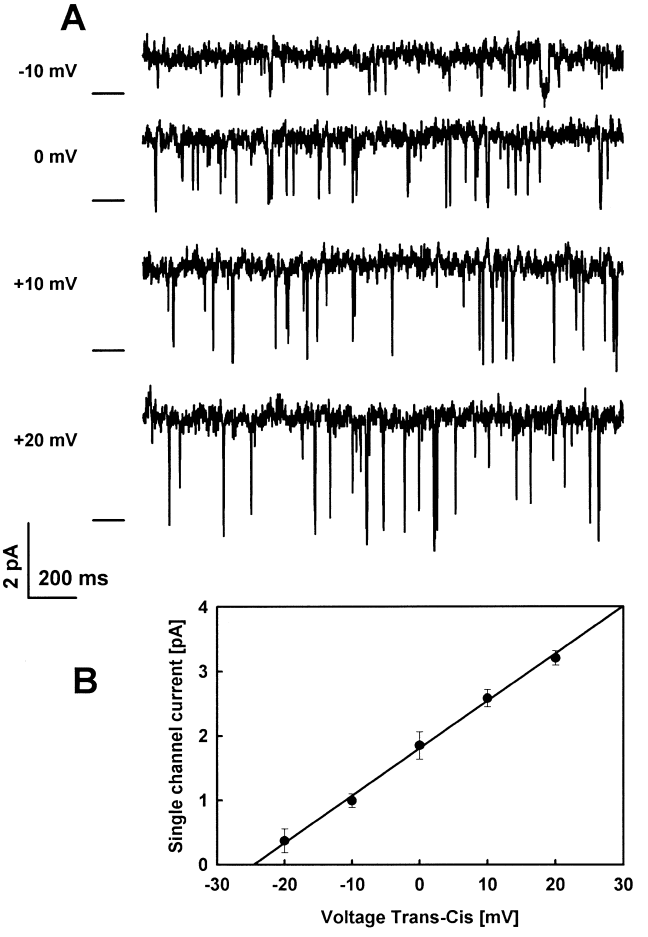
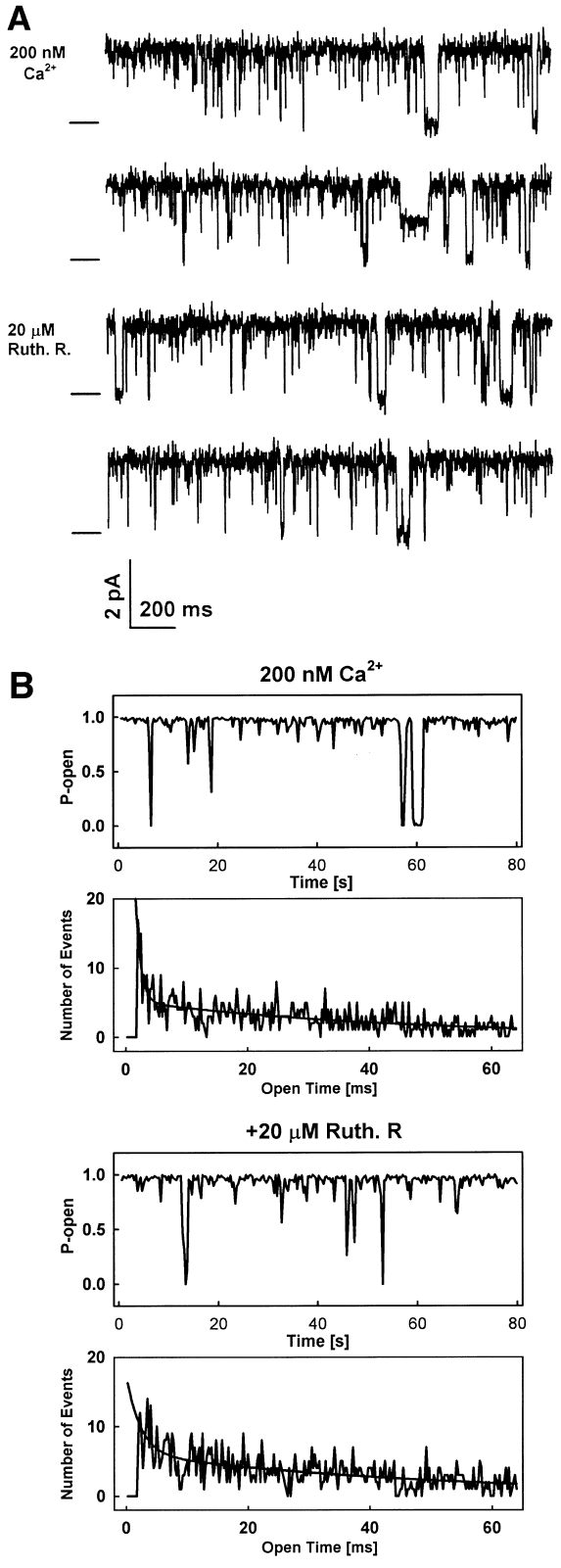


Fig. 3. (A) Voltage dependence of the single calcium channel current of microsomal membrane incorporated in BLM. Voltage is defined as *trans-cis*. Filter 200 Hz. The line on the left indicates the closed state of the channels. *Trans* solution: 55 mM Ca^{2+}. *Cis* solution: 110 mM KOH, 200 nM Ca^{2+}. (B) Current-voltage relation of the single calcium channel current. $E_{rev} = -24.6$ mV. Conductance 73 pS. $Ca^{2+}/K^{+} = 4.8$.



ities would be dangerous for living cells; it was therefore assumed that the endogenous inhibitor(s) of the observed channels were lost during microsome isolation.

The observed calcium channel selectivity, $Ca^{2+}/K^{+} = 4.8$, was rather poor in comparison to voltage dependent calcium channels (approx. 1000 for L-type [13]) or store operated channels (approx. 1000 in mast cells [14]). However, the selectivity was comparable to the published data for intracellular calcium release channels RYR ($Ca^{2+}/K^{+} = 6.5$; $Ba^{2+}/K^{+} = 5.8$) [11] and IP_3R ($Ba^{2+}/K^{+} = 6.3$) [15].

The regulation of intracellular Ca^{2+} in tracheal cells is known to involve Ca^{2+} release from intracellular stores. Ca^{2+} release from the endoplasmic reticulum in smooth muscle cells is mediated through both IP_3R and RYR channels [1–6,12,16,17] as suggested by measurements of Ca^{2+} release from intracellular stores or of Ca^{2+} activated chloride current in tracheal cells. However, single channel properties of calcium channels from tracheal microsomes have not been studied so far. Therefore, it was of interest to know whether our observed calcium channel had single channel properties of the known RYR or IP_3R calcium channels.

It was reported in our previous studies that type 1 and type 2 RYR calcium channels under our experimental conditions have conductances of 90–110 pS [18,19], are activated by MgATP and inhibited by ruthenium red (for a review, see [20]). The conductance of the tracheal calcium channel observed in our study was lower than that of the known RYR channels, and in addition, the calcium channel observed in our study was active at low *cis* Ca^{2+} concentrations; ATP did not activate it and ruthenium red did not block it. Similarly, concerning the IP_3R channel, it is known that the single type 1 and type 2 IP_3R

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Fig. 4. (A) The effect of ruthenium red on the single calcium channel current of microsomal membrane incorporated in BLM. The control *cis* solution contained 200 nM Ca^{2+}. The line on the left indicates the closed state of the channels. Filter 200 Hz. Same solutions as in Fig. 1. (B) Open probability ($n = 2378$ and 2785 events) and open time histograms ($n = 719$ and 899 events) of the single calcium channel at control conditions (200 nM Ca^{2+}, P-open = 0.91; mean open times: $\tau_1 = 0.8$ ms and $\tau_2 = 43$ ms) and after the addition of 20 μM ruthenium red (P-open = 0.92; mean open times: $\tau_1 = 2.3$ ms and $\tau_2 = 51$ ms).

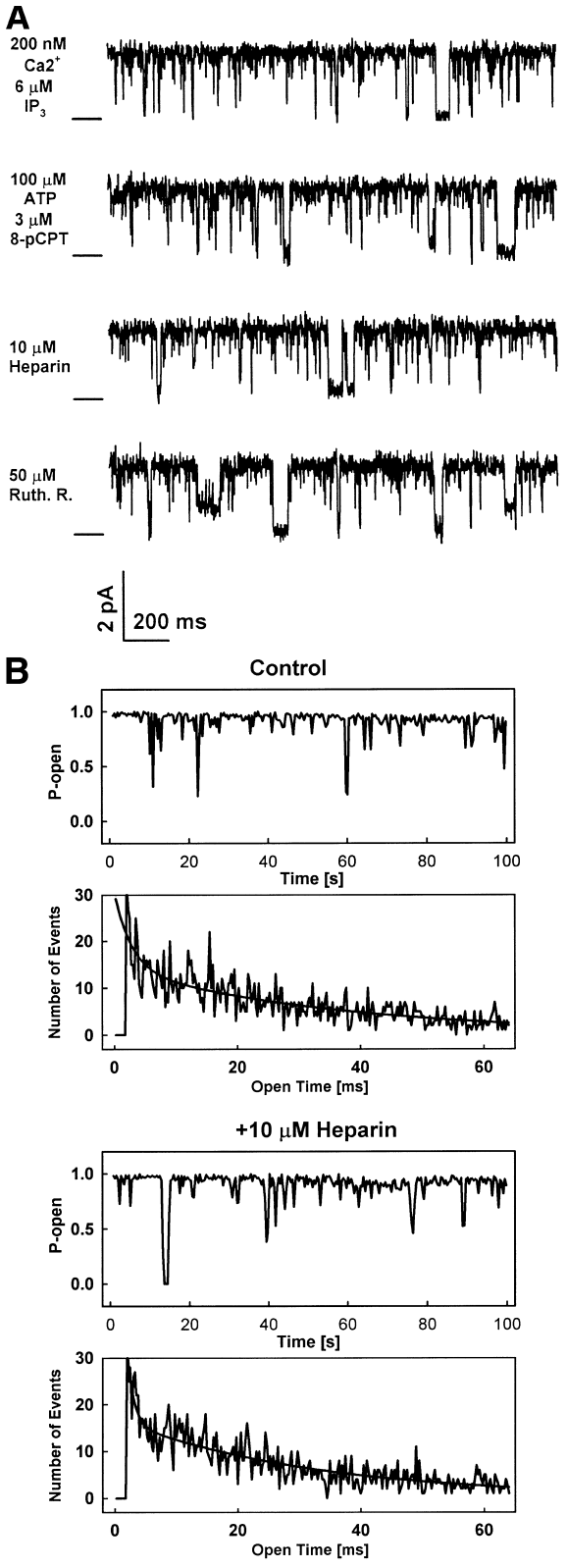

Fig. 5. (A) Effects of ATP, 8-pCPT-cGMP, heparin and ruthenium red on the single calcium channel current of microsomal membrane incorporated in BLM. The control *cis* solution contained 200 nM Ca^{2+} and 6 μM IP_3. 8-pCPT stands for 8-pCPT-cGMP. The line on the left indicates the closed state of the channels. Filter 200 Hz. Same solutions as in Fig. 1. (B) Open probability ($n = 4495$ and 4879 events) and open time histograms ($n = 1736$ and 1856 events) of the single calcium channel at control conditions (200 nM Ca^{2+}, 6 μM IP_3, *P*-open = 0.91; mean open times: $\tau_1 = 2.7$ ms and $\tau_2 = 37$ ms) and after the addition of 10 μM heparin (*P*-open = 0.90; mean open times: $\tau_1 = 1.1$ ms and $\tau_2 = 32$ ms).
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channels are activated by IP_3 and inhibited by heparin [8,21,22]. However, the calcium channel observed in our study was not inhibited by heparin and was active without addition of IP_3. From the presented results we suggest that the observed calcium channel is neither identical with type 1 or type 2 ryanodine calcium release channel, nor is the type 1 or type 2 IP_3 receptor calcium release channel. Further studies are needed to explain the origin and the modulators of this type of calcium channel.

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