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Long Ca^{2+} channel opening induced by large depolarization and Bay K 8644 in smooth muscle cells isolated from guinea-pig detrusor

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1 In smooth muscle cells enzymatically isolated from guinea-pig urinary bladder, Ca^{2+} channel currents were recorded by conventional cell-attached patch clamp techniques. In most recordings Bay K 8644 (2 μM) was contained in the patch pipette.

2 Closure of Ca^{2+} channels observed during the repolarizing steps was significantly slowed by preconditioning with large depolarizations (+80 and 100 mV), with or without Bay K 8644 in the pipette.

3 The sum of the unitary Ca^{2+} channel current traces obtained after large conditioning depolarizations (in the presence of Bay K 8644) showed a slowly deactivating tail current.

4 By use of this slow deactivating feature, the current-voltage relationship of the unitary Ca^{2+} channel current was continuously measured with a ramp pulse after large depolarization. The slope conductance ranged from 22 to 30 pS, compatible with that of L-type Ca^{2+} channels.

5 It is concluded that L-type Ca^{2+} channels in guinea-pig detrusor cells are open for much longer after large depolarizations consistent with their being two channel open states, and that Bay K 8644 prolongs the lifetime of both open states. The underlying mechanisms are discussed.

Keywords: Smooth muscle; guinea-pig urinary bladder; Ca^{2+} channels; Bay K 8644; dihydropyridines; channel kinetics

Introduction

Voltage-dependent Ca^{2+} channels are involved in many physiological functions. In the detrusor, it is thought that extracellular Ca^{2+} flowing through dihydropyridine-sensitive (L-type) Ca^{2+} channels plays a major role in the contraction of this smooth muscle including that evoked by neurotransmitters (summarised by Brading, 1987; Anderson, 1993).

Previously, we applied a whole-cell patch clamp technique to guinea-pig detrusor cells, and showed that even after 5 s of large conditioning depolarization (at +80 mV) the test currents were not inactivated, and the inward tail currents seen upon subsequent repolarization of the cell membrane to a holding potential deactivated very slowly (Nakayama & Brading, 1993a,b). These results could be explained by the presence of multiple open states of L-type Ca^{2+} channels, where the conformation of the Ca^{2+} channel is converted from the normal to a long channel open state during large depolarization. Similar phenomena, i.e. lack of inactivation during large depolarization and slow deactivation upon repolarization were also observed in the presence of a dihydropyridine Ca^{2+} channel agonist, and the slow deactivation of the tail current was more pronounced in the presence of the Ca^{2+} channel agonist (Brading & Nakayama, 1993; Nakayama & Brading, 1995a).

However it is well known that dihydropyridine Ca^{2+} channel agonists alone prolong channel opening of L-type Ca^{2+} channels during depolarization. This phenomenon is often explained by the mode gating mechanism ('mode 2' gating, Hess *et al.*, 1984). In cardiac myocytes, it has also been reported that β-adrenoceptor agonists and membrane permeable cyclic AMP analogues caused a 'mode 2'-like high activity gating mode and that the decay of the tail current was slow when L-type Ca^{2+} channels were in this gating mode (Yue *et al.*, 1990).

In the present study, using Ba^{2+} as a charge carrier and the cell-attached mode of the patch clamp technique, we measured unitary Ca^{2+} channel currents in guinea-pig detrusor cells. The closure of individual Ca^{2+} channels upon repolarization was slowed by preceding large depolarizing steps. Bay K 8644 in the patch pipette also slowed channel closing after test depolarization, and a preceding large depolarization caused a marked further slowing. Using the feature of slow deactivation, the voltage-dependence of the unitary Ca^{2+} channel current was also measured.

Methods

Cell preparation

Guinea-pigs (300-400 g) of either sex were stunned and bled. The urinary bladder was immediately dissected and its mucosa was carefully removed. Smooth muscle cells were enzymatically (0.08% collagenase, 0.1% pronase and 0.05% trypsin inhibitor) isolated as previously described (Nakayama, 1993). Some of the cell suspension was stored in a refrigerator and used for up to 6 h.

Electrical recording

A standard cell-attached patch clamp technique (Hamill *et al.*, 1981) was used to record unitary Ca^{2+} channel currents. The experiments were carried out at room temperature (22-26°C). When a Ba^{2+}-rich solution was used to fill the patch pipette, the pipette resistance was in the region of 5 MΩ. When single channel currents were measured, the seal resistance between the pipette and cell membrane was 10-20 GΩ. Capacitive surge was partially compensated electrically. Unless otherwise stated, the voltage of the patch membrane was clamped at −60 mV (+60 mV in amplifier). The membrane potential of the cell was assumed to be zero, when superfused with a high K^+, nominally Ca^{2+}-free solution.

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A cut-off frequency of ≈1.2 kHz (8 pole butterworth filter) was applied. Patch clamp amplifiers (Axopatch 1-B and Axopatch 200A, Axon Instruments, U.S.A.) were operated through an AD/DA converter (TL-1, Axon Instruments, U.S.A.), using an IBM-AT compatible computer (on-line recording). Computer programmes were written with AxoBasic Libraries (generous gift from Inter Medical, Nagoya Japan). In most experiments the dwell time of the sampling was set to be 50–100 μs. Current traces were drawn with an X-Y plotter (MP4200, Graphtech, Japan) in HP-GL mode.

Test voltage steps to +40 mV were applied with or without preconditioning large depolarizations (+80 or +100 mV). In our previous study using a whole-cell patch clamp mode in guinea-pig detrusor cells, it was found that the degree of activation was almost maximum at +20 mV (Nakayama & Brading, 1993a). In this study, to achieve maximal activation of Ca^{2+} channel, a greater potential (+40 mV) was used.

Drugs and solutions

The pipette solution had the following composition (mM): $BaCl_2$ 50, TEA-Cl 60, glucose 11.8, HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid)/Tris 11.8; pH adjusted to 7.4 at 24°C. In most experiments, the pipette solution also contained 2 μM Bay K 8644. The 'normal' bathing solution had the following composition (mM): NaCl 125, KCl 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 11.8, HEPES/Tris 11.8 (pH 7.4–7.5 at 24°C). The current recordings were made in Ca^{2+}-free, high-K^+ (100 mM) solution. The extracellular Na^+ was not completely replaced with K^+, because in smooth muscle intracellular pH progressively decreases in Na^+-free solutions (Nakayama *et al.*, 1994) and the fall of pH may consequently affect the kinetics (availability) of the Ca^{2+} channels (Klöckner & Isenberg, 1994). The composition of the solution was modified iso-osmotically.

(±)-Bay K 8644 (methyl 1, 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluorometrylphenyl)-pyridine-5-carboxylate) was purchased from Calbiochem (San Diego, U.S.A.). Its stock solution (1 mM, dissolved in ethanol) was kept cool and protected from light. The drug was diluted just before use. Collagenase was purchased from Wako Pure Chemical, (Japan); pronase from Fluka (Switzerland); trypsin inhibitor from Sigma (U.S.A.).

Statistics and data analysis

The numerical data were expressed as means ± standard deviation (s.d.). Curve fitting of the decay of the sum of unitary Ca^{2+} currents was done by fitting the discrete data points iteratively with an exponential function (Nakayama & Brading, 1993a).

Results

Long channel opening after large depolarization

In isolated detrusor cells, Ca^{2+} channel currents were measured using a standard cell-attached patch clamp technique. The cells were superfused with Ca^{2+}-free, high-K^+ solutions and the patch pipette contained 50 mM Ba^{2+} as a charge carrier. A holding potential of −60 mV was applied to the patch membrane. Test potentials (+40 mV, 20 ms) were applied at 30 s intervals. A conditioning step of +80 mV (5 s) was applied before every other test potential (+40 mV), to rule out systematic errors. In Figure 1a(i), with no Ca^{2+} channel agonist in the pipette, switch off of unitary Ca^{2+} currents after simple depolarization was very rapid and therefore was masked by the capacitive surge on repolarization. In contrast, after preconditioning depolarizations unitary Ca^{2+} channel currents were transiently observed (Figure 1a(ii)). Using the same paired protocol repeated five times, there was no charge movement after 10 ms in the repolarizing step without conditioning depolarization, but after conditioning, the total charge movement was 0.028 ± 0.03 pC.

When the patch pipette contained Bay K 8644 (2 μM), after a simple depolarization was applied, unitary Ca^{2+} channel currents were seen in the early stage of the repolarizing step, but hardly ever after 50 ms (Figure 1b(i)). On the other hand, pre-conditioning depolarizations resulted in a significant delay of channel closure (Figure 1b(ii)). In the same paired protocol repeated five times, the total charge movement after 10 ms in the repolarizing step was 0.86 ± 0.22 pC, 14.2 ± 3.9 times greater than without conditioning. Qualitatively similar results were obtained in all experiments in which unitary Ca^{2+} channel currents were observed.

Figure 1c(i) and (ii) summarizes the effects of large conditioning depolarization in the presence and absence of Bay K 8644, respectively. The number of the current traces (sweeps) is plotted against the time taken for all the channels to close in the repolarizing step (in most recordings more than one channel is included in the patch membrane). In the absence of Bay K 8644 conditioning depolarization caused a clear and significant prolongation of the time taken for channel closure, but in all of the traces all channels had closed before 50 ms. In the presence of the Ca channel agonist channel closure after 50 ms was seen on repolarization from the test step both with and without conditioning depolarization, although the number closing beyond 50 ms was significantly increased after conditioning.

Sum of unitary Ca^{2+} channel currents

In Figure 2, in the presence of Bay K 8644 preconditioned depolarizations were repeated six times at 30 s intervals: the preconditioning step at +80 mV (5 sec), test step at +40 mV (20 ms). In one of the six current traces we did not observe an open Ca^{2+} channel, when the cell membrane was returned to the holding potential, at least after the first 5 ms. In Figure 2a an example of a trace with open channels (indicated by an asterisk) is superimposed on one in which no channel was open beyond 5 ms (null sweep). The current traces in Figure 2b were obtained after subtracting the null trace from each one. The patch pipette contained more than four Ca^{2+} channels and after conditioning by large depolarization the channels were open for a considerable time even during the repolarizing step at −60 mV.

Capacitive currents upon repolarization have subtle differences in shape, depending on whether preconditioning depolarizations are applied. When accumulating the current traces, the deformity from these differences becomes significant. Thus, subtraction between unconditioned and conditioned currents was not normally done.

The current trace shown in Figure 2c was constructed by adding the five current traces in Figure 2b. These results clearly demonstrate the formation of the slow deactivating tail current after large conditioning depolarization (Nakayama & Brading, 1993a; 1995b). The decay time constant of the summed tail current was 26 ms. The shape of the decay of the accumulated tail current was not smooth at the beginning of the repolarizing step, because the null-sweep trace (subtracted from the other traces) contained transient channel openings during the first 5 ms of the repolarization.

The patch pipette occasionally contained more than 20 Ca^{2+} channels. If the same pulse sequence shown in Figure 2 was applied, the tail current seen upon repolarization to the holding potential decayed very slowly after a large preconditioning depolarization (data not shown), being similar to that shown Figure 2c.

Voltage-dependence of Ca^{2+} channel current amplitude

To examine how the amplitude of the unitary Ca^{2+} channel current changes with membrane potential, the voltage of the repolarizing step was changed. Figure 3 shows an example of such an experiment. All pulse sequences were preceded by 5 s

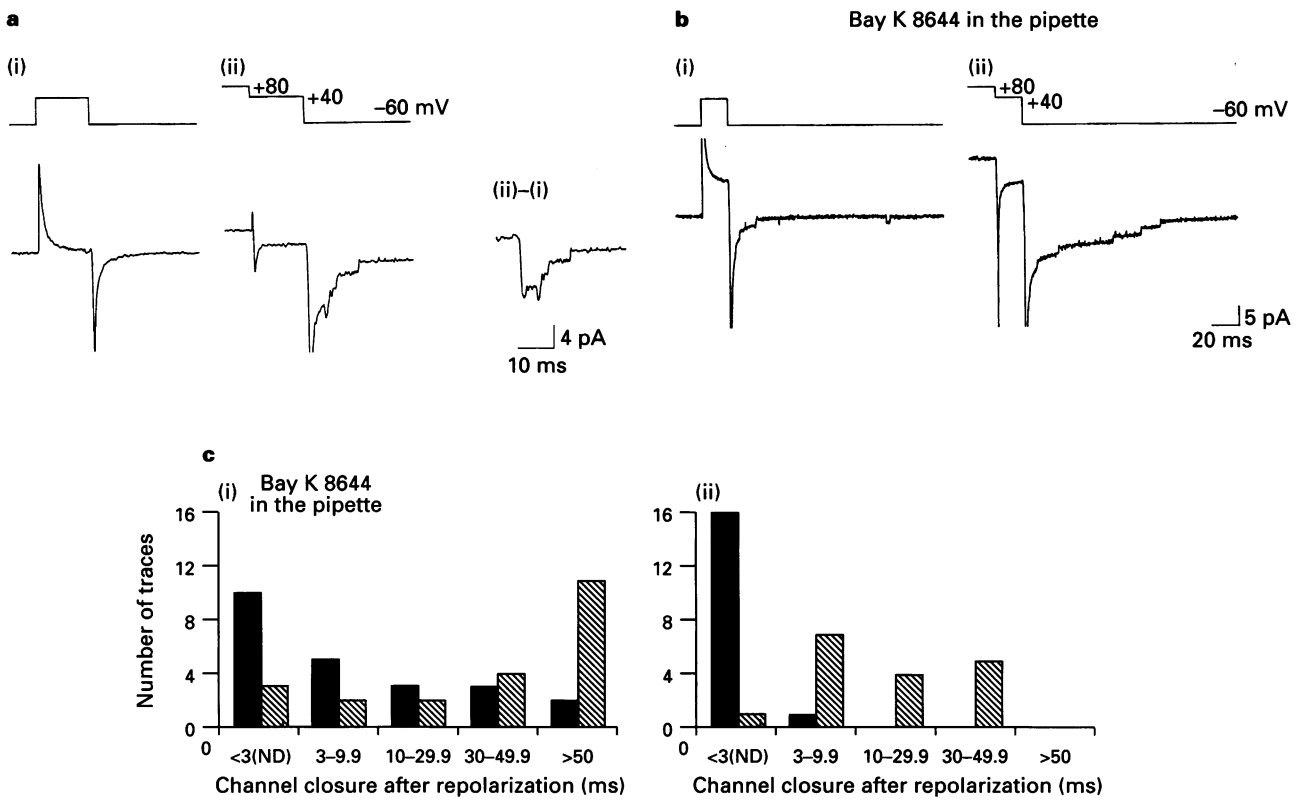

Figure 1 Cell-attached recording of the Ca^{2+} channel currents. Pipette contained 50 mM Ba^{2+}. Cells were superfused with Ca^{2+}-free, high-K^+ solution. The holding potential was −60 mV. (a) Effect of the pre-conditioning step on the inward tail current without Bay K 8644 in the patch pipette. In (i) the patch membrane was depolarized with a simple test potential (+40 mV, 15 ms). In (ii) the membrane was depolarized to +80 mV for 5 s before the test potential. Only the last 20 ms of the conditioning step is shown in the current trace. The capacitive surge upon repolarization to the holding potential (i) was subtracted from (ii). In (ii)−(i), only the current trace in the repolarizing step is shown. (The artifact in the test step brought about by the subtraction is not shown). (b) As in (a) but with 2 μM Bay K 8644 in the patch pipette. (c) Channel closure in the repolarizing step at −60 mV in the presence (i) and absence of 2 μM Bay K 8644 (ii). The same paired pulse sequence shown in (a) was used. The current recordings were analysed only when at least one channel opening was recognised in the sequentially applied paired pulses. In the absence of Ca^{2+} agonist, 17 paired recordings were analysed in 10 cells, while in the presence of Bay K 8644, 23 recordings in 14 cells. In most experiments, the patch membrane contained several (sometimes more than 12) Ca^{2+} channels. The number of the current traces (sweeps) is plotted against the time taken for all the channels to close in the repolarizing step. Solid and hatched columns correspond to data obtained by simple and conditioned depolarizations, respectively.

conditioning depolarization at +80 mV. The membrane patch contained one channel. The voltage of the repolarizing step was changed from −40 to −80 mV. For each repolarizing potential, the pulse sequence was repeated five times, and at least one null sweep trace (no channel opening) was obtained. The current traces in the figure were obtained by the same procedures shown in Figure 2a and b. The amplitudes of the resultant unitary Ca^{2+} currents were 2.2 pA at −40 mV, 2.8 pA at −60 and 3.3 pA at −80 mV. Similar experiments were performed in three other cells. In one of the experiments, the patch membrane included many (more than six) Ca^{2+} channels. When the voltage of the repolarizing step was changed from −40 to −60, −80 and −100 mV, the product of the number of the channel and open probability (NP_o) decreased to 60.2, 36.2 and 27.6% of that at −40 mV, respectively.

Figure 4 shows voltage-dependence of the amplitude of the unitary Ca^{2+} channel current. The patch pipette contained Bay K 8644 (2 μM). After a large conditioning depolarization at +80 mV (4 s), a ramp pulse (+80 to −80 mV) was applied to continuously measure the *I-V* relationship. The same voltage sequence was repeated many times in the same membrane patch. It may be noteworthy that the long channel opening induced by large depolarization made this type of experiment possible. Null sweep traces were subtracted from the current traces which contained channel opening. The resultant slope conductance (obtained from eleven cells) ranged from 22 to 30 pS. (Similar slope conductance was obtained even in the absence of Ca^{2+} agonist.) Also, when a patch pipette contained many Ca^{2+} channels, the same ramp (+80 to −80 mV) shown in Figure 4 was applied after large depolarization. Ca^{2+} channels successively closed as the membrane potential was repolarized (data not shown).

Discussion

In smooth muscle cells isolated from guinea-pig urinary bladder, we have previously shown using whole-cell voltage clamp techniques that the Ca^{2+} channels present are mainly L-type (Nakayama & Brading, 1993a). We have also shown that the amplitude of the Ca^{2+} channel current evoked at the test potential (+40 mV) was not significantly decreased after pre-conditioning with large depolarizing steps (at +80 mV) for up to 5 s (Nakayama & Brading, 1993b), and that the tail currents induced by repolarizations to the holding potential were progressively increased by prolonging the preconditioning depolarization (Nakayama & Brading, 1993a). Thus, we have proposed that large depolarizations switch the Ca^{2+} channel from its normal open (O_1) to a long open state (O_2). Furthermore, prolongation of closure of Ca^{2+} channel current after large depolarization was also observed in the presence of Ca^{2+} channel agonist (Nakayama & Brading, 1995a). This could be similarly explained by the presence of O_1* and O_2* (asterisks indicate states with Ca^{2+} agonist bound).

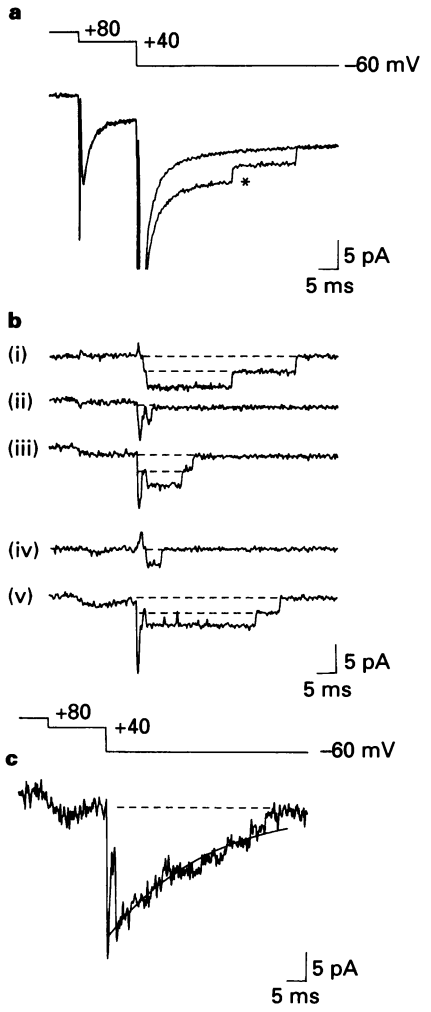

Figure 2 Repeat of conditioned depolarizing steps. Conditioned depolarizations (conditioning step, +80 mV for 5 s; test step, +40 mV for 20 ms) were repeated at 30 s intervals. The patch pipette contained 2 μM Bay K 8644. In (a), a null current trace (sweep) and an example of a trace with open channel (indicated by asterisk) are superimposed. Only the last 20 ms of the conditioning step is shown. The null current trace was subtracted from five traces in which channels were open, and the resultant open traces are shown in (b). The subtracted current trace (i) is a result using the traces shown in (a). In (c) the sum of the five subtracted current traces (b, (i) to (v)) are shown. The decay of the Ca^{2+} channel current in the repolarizing step was iteratively fitted with a single exponential function: $I(t) = A_0 + A_1 \exp(-t/\tau)$.

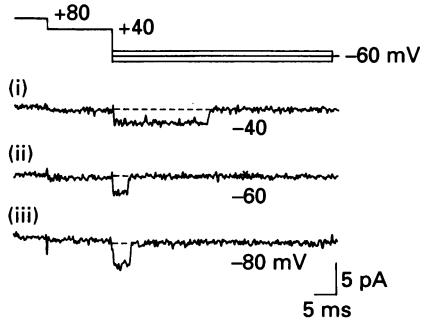

Figure 3 Effects of changing the voltage of the repolarizing step. The repolarizing potential (the third step) was changed from −40 to −80 mV. The pulse sequence shown was preceded by a 5 s conditioning depolarization (at +80 mV). Each repolarizing potential was applied five times, and at least one of the current traces obtained at each repolarizing potential was a null current trace. The current traces (i) to (iii) were obtained by the same subtracting process shown in Figure 2(a) and (b). Bay K 8644 (2 μM) was included in the pipette.

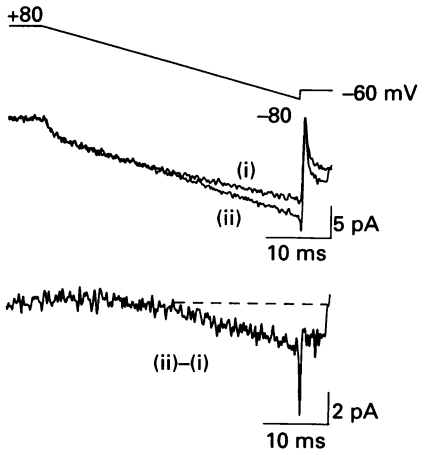

Figure 4 Unitary current-voltage relationship obtained using a ramp pulse (+80 to −80 mV) preceded by a conditioning depolarization (+80 mV, 4 s). The patch pipette contained 2 μM Bay K 8644. The slope of the subtracted current trace ((ii)−(i)) corresponds to the *I-V* relationship of the unitary Ca^{2+} channel current.

In the present study, using the cell-attached mode of the patch clamp technique, we examined the effects of large conditioning depolarizations (mainly in the presence of Bay K 8644) on the behaviour of the unitary Ca^{2+} channel currents. Either in the presence or absence of this dihydropyridine Ca^{2+} channel agonist, closure of the Ca^{2+} channels upon repolarization to the holding potential was significantly delayed after large conditioning depolarizations (Figure 1). Especially, in the presence of Bay K 8644, a considerable number of the channels were continuously open even after 100 ms. The deactivation time constant of the summed unitary Ca^{2+} channel current shown in Figure 2c was 26 ms. This value is presumed to correspond to the time constant (30–50 ms) of the whole-cell tail current after large depolarization in the presence of Bay K 8644 (Nakayama & Brading, 1995a), although the cell-attached patches in the present paper are not directly comparable, due to the different solutions used, and the uncertainties about the intracellular environment during cell-attached recordings.

From computer fitting of the current-voltage relationship of the peak inward current (whole-cell experiments, data not shown), the voltage-dependence of the degree of activation ($d_\infty(E)$) should be maximal at the test potential used (+40 mV). Thus, the prolongation of the unitary tail currents is not simply explained by a further increase in the degree of activation during depolarization to +80 mV.

In cardiac cells it has also been reported that Bay K 8644 induces sizeable tail current after simple depolarizations accompanied by a hyperpolarizing shift of the activation curve. Both of the tail current formation and hyperpolarizing shift of activation curve could be explained by the slower transition rate of the open to closed states in the presence of Bay K 8644 (Bechem & Hoffmann, 1993). (The open and closed states may correspond to O_1* and C* in the present study). In guinea-pig detrusor cells, in the presence of Ca^{2+} agonists we also observed whole-cell tail currents after simple depolarizing steps and a significant hyperpolarizing shift of the activation curve. However, even in the presence of Bay K 8644, the time constant (2.5–5 ms) of the whole-cell tail current after simple depolarizing step was significantly smaller than that (30–50 ms) after large conditioning depolarization (Nakayama & Brading, 1995a).

After large conditioning depolarizations, Ca^{2+} channels showed prolonged opening in the repolarizing step. Thus, we applied a ramp pulse to measure the *I-V* relationship of unitary Ca^{2+} channel amplitude (Figure 4). The unit amplitude changes almost linearly when the membrane potential was below 0 mV, although due to its low signal-to-noise level, the *I-V* relationship was not clear near the reversal potential. The linear *I-V* relationship is consistent with that measured in whole-cell tail currents after large depolarizations (Figure 9 in Nakayama & Brading, 1993a). The slope conductance of the unitary Ca^{2+} channel current ranged from 22 to 30 pS. Among the voltage-sensitive Ca^{2+} channels already described (Spedding & Paoletti, 1992), the values obtained in the present study correspond to that of the L-type Ca^{2+} channel seen in heart and smooth muscles (Hess *et al.*, 1986; Benham *et al.*, 1987).

Conditioning depolarization of 5 s duration is often used to achieve steady state inactivation of the Ca^{2+} channels. In the present study, unitary tail currents were, in fact, enhanced (prolonged opening) rather than depressed after preconditioning depolarization at +80 mV (Figure 1). Since Ca^{2+} channels were open at the beginning of the repolarizing step (Figure 2b and Figure 3), the delayed closure of Ca^{2+} channels is not explained by a reversed transition from an inactivated state ($I \rightarrow O \rightarrow C$ or $I^* \rightarrow O^* \rightarrow C^*$). Also, when many Ca^{2+} channels were included in the patch membrane, inward current was clearly observed in the test step (+40 mV) even after large conditioning depolarization (data not shown). Further, when a ramp pulse (from +80 to −80 mV) was applied after large depolarization, the amplitude of the unitary Ca^{2+} current gradually and continuously increased with repolarization, and in a membrane patch which included several Ca^{2+} channels, it was observed that Ca^{2+} channels successively closed as the membrane potential was repolarized. Thus, it seems unlikely that the large depolarization-induced slow-tail current in guinea-pig detrusor smooth muscle cells is due to another subset of Ca^{2+} channels reprimed by large and long depolarizations which open upon repolarization of the cell membrane, as seen in cultured skeletal muscle cells (Fleig & Penner, 1995).

Taken together, these results support our previous hypothesis that Ca^{2+} channel agonists can induce long channel opening ($O \rightarrow O^*$), and that during large depolarization in the presence or absence of Ca^{2+} channel agonists the conformation of L-type Ca^{2+} channels in guinea-pig detrusor cells is transferred into a second open state (O_2 and O_2^*) in which Ca^{2+} channels inactivate very slowly or not at all during the depolarizing steps (Nakayama & Brading, 1993b) and deactivate slowly upon repolarization (Nakayama & Brading, 1993a; 1995a). The presence of the second open state induced by large depolarization may explain non-inactivating Ca^{2+} currents seen when the cell membrane was depolarized to high positive potentials (at which the contribution of window current is small due to its voltage-dependency) (Aaronson *et al.*, 1988; Nakayama & Brading, 1995b).

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