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Effects of phosphorylation-related drugs on slow Ca^{2+} tail current in guinea-pig detrusor cells

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Abstract

In isolated guinea-pig detrusor cells, large conditioning depolarizations evoke slowly deactivating Ca^{2+} tail currents, considered to represent the second open state. The possible involvement of channel phosphorylation in this open state was examined. Application of isoprenaline caused a marginal increase in Ca^{2+} channel current evoked by simple depolarization, while forskolin did not. During application of either drug, slow-tail currents were never observed after simple depolarizations. The conditions necessary to induce slow-tail currents were not changed, even when cyclic AMP, ATP-γ-S (adenosine 5′-*O*-(3-thiotriphosphate)), GDP-β-S (guanosine 5′-*O*-(2-thiodiphosphate)) (in the pipette) or H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) (to the bathing solution) was applied. The frequent depolarization protocol, known to facilitate Ca^{2+} current via Ca^{2+} and cyclic AMP-dependent phosphorylation mechanism(s) in cardiac myocytes, did not induce slow-tail currents. These results suggest that the transition of Ca^{2+} channels to the second open state during large depolarization is not a result of (voltage-operated) channel phosphorylation itself. Possible underlying mechanisms are discussed.

Keywords: Smooth muscle; Urinary bladder, guinea-pig; Ca^{2+} channel; β-Adrenoceptor; Phosphorylation; Open state

1. Introduction

In guinea-pig urinary bladder smooth muscle cells, Ca^{2+} channels are mainly of the L-type. Previously, we have reported that slowly deactivating Ca^{2+} tail currents are observed after large conditioning depolarizations (Nakayama and Brading, 1993a). This phenomenon is interpreted as that the conformation of the Ca^{2+} channel is transferred from the normal to a second open state (O_2) during large depolarization. Due to its non-inactivating (or very slowly inactivating) nature (Nakayama and Brading, 1993b), Ca^{2+} channels in the O_2 state may make an important contribution to persistent Ca^{2+} influx (Nakayama and Brading, 1995b), which is thought to maintain slow and tonic contractions, characteristics of smooth muscle. Recently, single channel recordings corresponding to the slowly deactivating tail current have been seen (Nakayama and Brading, 1996).

The open time of L-type Ca^{2+} channel is prolonged by so-called dihydropyridine Ca^{2+} channel agonists (Hess et al., 1984). In cardiac myocytes, it has been shown that Bay K 8644 slowed the deactivation time course of the tail current (e.g., Lacerda and Brown, 1989). Thus, we examined the interaction of large conditioning depolarization and Ca^{2+} channel agonists in detrusor cells. However, it was found that the time constants of the tail currents induced by large depolarization and Ca^{2+} channel agonists were different. Furthermore, the decay time course was additively slowed by the combination of the two factors. These suggest that the mechanisms underlying the slow-tail current seen after large depolarization are distinct from those reported for Ca^{2+} channel agonists (Brading and Nakayama, 1993; Nakayama and Brading, 1995a).

In neuronal and muscular cells, it has been suggested that large conditioning depolarizations potentiate the subsequent test (L-type) Ca^{2+} channel currents through a voltage-dependent phosphorylation mechanism (e.g., Artalejo et al., 1992; Sculptoreanu et al., 1993). In smooth muscle, there are many papers published concerning phosphorylation-induced modulation in Ca^{2+} channel current

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(reviewed by McDonald et al., 1994; Xiong and Sperelakis, 1995). Thus, in the present study, we examined whether phosphorylation has a role in the formation of the slow tail current and the second open state of the Ca^{2+} channels.

2. Materials and methods

2.1. Preparation of cells

Guinea-pigs (300–400 g) of either sex were stunned and bled, and the urinary bladder was immediately dissected. Smooth muscle cells were enzymatically isolated (0.08% collagenase, 0.1% pronase and 0.05% trypsin inhibitor) as previously described (Nakayama and Brading, 1993a,b).

2.2. Ca^{2+} current recording

A standard patch clamp technique (Hamill et al., 1981) was used to record macroscopic Ca^{2+} channel currents. Patch clamp amplifiers (Axopatch 200A, Axon, USA; EPC-7, List, Germany) were operated through AD/DA converters (TL-1, Axon, USA; DT2801A, Data Translation, UK), using IBM-AT compatible computers. The resistance of the patch pipette was 2.5–5 MΩ, when a Cs^+-rich pipette solution was used. After rupture of the cell membrane, the series resistance was normally < 10 MΩ. Smooth muscle cells used had a membrane capacitance of 30–80 pF. The capacitive surge was electrically compensated, and the series resistance was partially compensated (by 50–70%). A cut-off frequency of 5 kHz was applied to reduce the noise. Unless otherwise described, the cell membrane was clamped at −60 mV (the holding potential). The experiments were carried out at room temperature (22–26°C). The decay time course of the tail currents observed after large conditioning depolarizations was fitted with a single exponential function (Nakayama and Brading, 1993a).

2.3. Solutions and chemicals

The normal bathing medium had the following composition (mM): NaCl, 125; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 11.8 and Hepes, 11.8; pH was adjusted to 7.4 with Tris base. When whole-cell Ca^{2+} currents were recorded, K^+ was replaced with the equimolar Cs^+ to rule out contribution of inward K^+ current upon returning the cell membrane to the resting potential. The composition of the normal pipette solution was (mM): CsCl, 141; $MgCl_2$, 1.4; EGTA (ethyleneglycol-bis-(β-aminoethylether) *N,N,N′,N′*-tetraacetic acid), 2; ATP, 1; GTP, 0.1; TEA (tetraethylammonium), 7; Hepes/Tris, 10 (pH 7.2). In some experiments, ATP-γ-S (adenosine 5′-*O*-(3-thiotriphosphate)) was used instead of ATP. Also, when the possible involvement of G-proteins was examined, 1 mM GDP-β-S (guanosine 5′-*O*-(2-thiodiphosphate)) was added to the pipette solution.

The following chemicals, drugs and enzymes were used in the present study: ATP (disodium salt), GTP (trisodium salt), cyclic AMP (free acid), H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) from Seikagaku Kogyo (Tokyo, Japan); (±)-isoprenaline-hydrochloride, ATP-γ-S (tetralithium salt), GDP-β-S (trilithium salt), EGTA (free acid), trypsin inhibitor (type 1-S) from Sigma (St. Louis, USA); collagenase, forskolin from Wako (Osaka, Japan); pronase (from *Streptomyces griseus*) from Fluka.

2.4. Statistics

Numerical data are expressed as mean ± standard deviation. When differences between means were evaluated by paired *t*-tests, a *t*-value of < 0.05 was taken as a statistically significant difference.

3. Results

3.1. Slow deactivating tail current

In isolated urinary bladder cells, whole-cell Ca^{2+} channel currents were recorded as previously described (Nakayama and Brading, 1993a,b), except replacing external K^+ with Cs^+. Under the superfusion of the Cs^+-containing solution, preconditioning steps at +80 mV (3–5 s) significantly increased the amplitude of the tail current and slowed the deactivation time course upon returning the cell membrane to the holding potential (−60 mV) (see Fig. 4A), as seen in previous experiments. Also, during the preconditioning large depolarization, the test inward currents (at 0 mV) were not depressed, but rather potentiated. These phenomena can be explained that during large depolarization, Ca^{2+} channels (L-type) are transferred from the normal (O_1) to a second open state (O_2) in which Ca^{2+} channels do not inactivate (or only very slowly inactivate), and deactivate much more slowly than in the O_1 state (Nakayama and Brading, 1993a). Slow deactivating Ca^{2+} tail currents are thus considered to represent the development of the O_2 state during large depolarizations.

3.2. Effects of cyclic AMP-related drugs

In some smooth muscles (e.g., porcine coronary artery, Fukumitsu et al., 1990; A7r5 cells, Marks et al., 1990) isoprenaline is known to potentiate Ca^{2+} channel current possibly through cyclic AMP-dependent systems, as seen in cardiac muscle. To test the possible contribution of a phosphorylation mechanism on the slowly deactivating tail currents, we first examined the effect of isoprenaline. Fig. 1 shows an example of one such experiment. Depolarizing

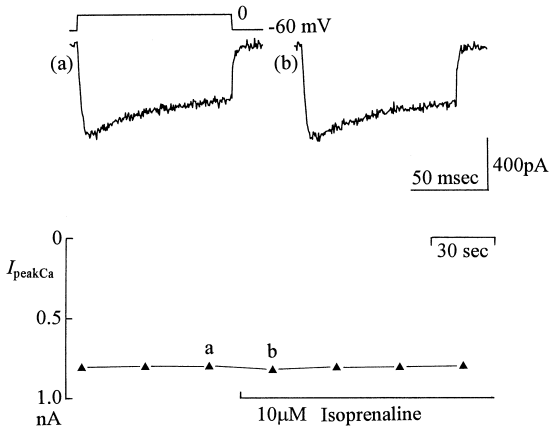

Fig. 1. An example of the effects of isoprenaline (10 μM) on Ca^{2+} channel current. Simple depolarization (0 mV, 100 ms) was applied every 30 s. The whole-cell current traces obtained in control medium and in the presence of isoprenaline are shown. The graph shows changes in the peak amplitude of the depolarization-mediated inward current.

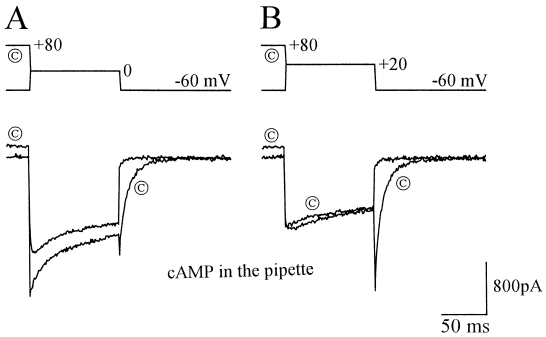

Fig. 2. Effects of cyclic AMP (100 μM) in the patch pipette. Unconditioned and conditioned test steps were applied. When conditioned, a depolarization step of +80 mV was applied for 5 s prior to the test step (100 ms). Only the last 25 ms of the conditioning step are shown in the figure. The test potential was 0 and +20 mV in (A) and (B), respectively. Conditioned current is indicated with ©.

steps to 0 mV (100 ms duration) were repeated at 30 s intervals. When 10 μM isoprenaline was applied to the recording medium, the amplitude of the peak inward current was very slightly, but statistically, significantly increased temporarily (by 4 ± 1%, $n = 4$) [see Fig. 1, current trace (b)]. In another cell, application of 30 μM isoprenaline increased the amplitude of the peak inward current by 6%. However, unlike large depolarization, application of isoprenaline never induced a slow-tail current.

Similar experiments were carried out with forskolin, a direct activator of adenylate cyclase. Forskolin (1 μM) was applied to three cells. In one cell, the amplitude of the peak inward current increased by 10%, while in the other two experiments, it decreased successively during the experiments. When 5 μM forskolin was used in two other cells, no potentiation was observed. As with isoprenaline, the bath applications of forskolin never induced slow-tail currents after depolarizing steps to 0 mV for 100 ms. Even in the presence of forskolin, a slow-tail current was induced only after large conditioning depolarization at +80 mV (5 s). The deactivation time constant upon repolarization to −60 mV ranged 7–10 ms in three cells.

The effect of preconditioning large depolarization was also examined with cyclic AMP (100 μM) itself in the patch pipette. In all six cells examined, a slow-tail current was not induced by a depolarization step to 0 mV (50–100 ms), up to 20 min after rupture of the cell membrane. As shown in Fig. 2, slow-tail currents were observed only after preconditioning depolarization (+80 mV, 5 s, indicated with ©). The decay time constant (τ_d) of the slow-tail current was 10.0 ± 2.4 ms ($n = 12$, Table 1A: the paired pulse protocol was applied 12 times in six cells). On the other hand, when the preconditioning large depolarization was not applied, the amplitude of the tail current was significantly smaller (16.2 ± 8.4%) than that after large conditioning depolarization. Also, its decay time constant (3.7 ± 2.5 ms) was normally significantly smaller, but had a wide range of distribution compared to its mean value. The relatively wide distribution of the decay time constant without preconditioning depolarization is presumably because the amplitude of the tail current is too small to precisely analyse (in curve-fitting), and also because a small population of Ca^{2+} channels may be transferred into the second, long channel open (O_2) state even during ordinary test depolarizations.

When the voltage of the test step was 0 mV (100 ms), the peak amplitude of the Ca^{2+} channel current was significantly larger (43 ± 9%) after preconditioning depolarization (Fig. 2A). When the positivity of the test depolarization step was increased to +20 mV, at which potential the degree of activation is considered to reach the maximum, the peak amplitudes were nearly the same in the conditioned and unconditioned test currents (B). Note that during large conditioning, depolarization Ca^{2+} chan-

Table 1
Effects of various drugs on the amplitude and deactivation time constant (τ_d) of tail current upon repolarization to −60 mV

	Conditioned	Unconditioned
(A) 100 μM cAMP in the pipette (n = 12)		
Amplitude (%)	100	16.2 ± 8.4#
τ_d (ms)	10.0 ± 2.4	3.7 ± 2.5#
(B) 100 μM H-7 in the bath (n = 7)		
Amplitude (%)	100	20.3 ± 12.2#
τ_d (ms)	9.2 ± 1.7	2.5 ± 2.3#
(C) 1 mM GDP-β-S in the pipette (n = 6)		
Amplitude (%)	100	22.3 ± 8.1#
τ_d (ms)	12.3 ± 3.0	2.8 ± 1.6#

Unconditioned and conditioned test steps were applied. When conditioned, a depolarization step of +80 mV was applied for 5 s prior to the test step (100 ms). The amplitude of the unconditioned tail current is expressed relative to that of the other tail current evoked after preconditioning depolarization.
#$P < 0.05$ compared with the conditioned tail current.

nel current was little inactivated in the current trace indicated with © in (B). The preconditioning large depolarization causes maximal activation of Ca^{2+} channels, while the test step of 0 mV causes only 60–70% of activation (Nakayama and Brading, 1993b). After large conditioning depolarization, the increase in the amplitude of the subsequent test current in (A) is due to lack of, or negligible degree of inactivation during the conditioning step, and also due to that the degree of activation provided by the conditioning depolarization (+80 mV) is larger than that of the test step (0 mV).

Similar paired pulse protocols used in Fig. 2 were applied, when the extracellular Ca^{2+} was substituted with the equimolar Ba^{2+}. Even in Ba^{2+}-containing solution, slow-tail currents ($\tau_d = \sim 12$ ms) were observed only after large conditioning depolarizations.

3.3. Effects of ATP analogue and protein kinase inhibitor

Thiophosphorylation product (phosphorylated with ATP-γ-S) is known to be resistant to enzymatic hydrolysis (Gratecos and Fischer, 1974). If the slow-tail current (and the transition of the Ca^{2+} channel to O_2 state) is a result of channel phosphorylation via a voltage-dependent phosphorylation mechanism (Artalejo et al., 1992) during large conditioning depolarization, we would expect that when ATP-γ-S is in the pipette, once a slow-tail current is induced by a large conditioning depolarization, all Ca^{2+} channel currents subsequently evoked by depolarizing pulses (even with no preconditioning large depolarization) are accompanied with slow-tail currents. We examined this possibility in the experiment shown in Fig. 3. The pipette solution contained 1 mM ATP-γ-S, and ATP was removed. Test depolarizing steps at 0 mV (100 ms) were repeated at 30 s intervals. The inward currents indicated with asterisks were preceded by a large conditioning depolarization to +80 mV (5 s). After the large conditioning depolarization, the peak amplitude of the inward current on the test step (b) was significantly increased [by about 40% compared to the previous Ca^{2+} current (a)]. This increase in the test inward current is again, presumably because large preconditioning depolarizations provide a maximal degree of activation, and because Ca^{2+} channels do not inactivate during these preconditioning steps. Also, after large conditioning depolarization, a slow-tail current was induced by the subsequent repolarization to the holding potential [Fig. 3, current trace (b)]. The Ca^{2+} channel current (c) was evoked by the subsequent unconditioned depolarization to 0 mV [30 s after (b)]. Note that the effect of the large conditioning depolarization observed in (b) was not retained in (c): no slow-tail currents were seen upon repolarization even in the presence of ATP-γ-S.

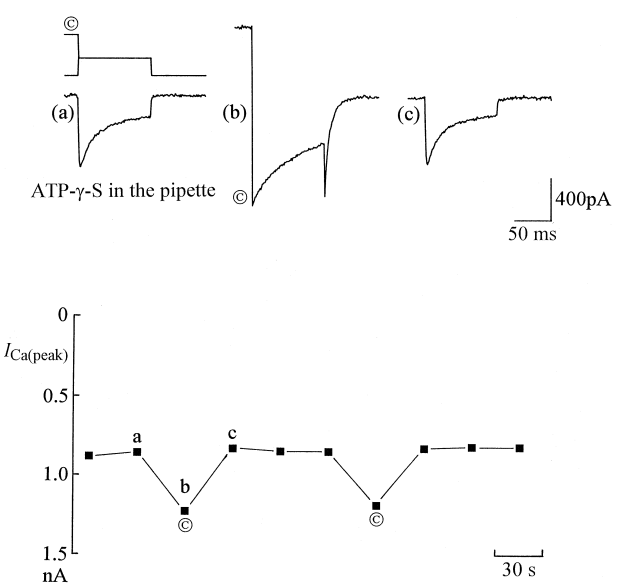

Fig. 3. Effects of ATP-γ-S in the pipette. Instead of ATP, 1 mM ATP-γ-S was contained in the patch pipette. Test depolarization to 0 mV was repeated at 30 s intervals. In the current trace (b), the test depolarization was preceded by a conditioning depolarization at +80 mV (5 s). The graph shows changes in amplitude of the peak inward current during the test step. © indicates application of preconditioning depolarization. (a)–(c) in the graph correspond to those in the current traces.

Table 2
Changes in the amplitude and time constant (τ_d) of the tail currents induced by the set of three consecutive depolarizations (unconditioned–conditioned–unconditioned) in the presence of 1 mM ATP-γ-S in the pipette ($n = 6$)

	Unconditioned (former)	Conditioned	Unconditioned (subsequent)
Amplitude (%)	$19.1 \pm 7.5^{\#}$	100	$19.8 \pm 5.8^{\#}$
τ_d (ms)	$1.8 \pm 1.1^{\#}$	8.2 ± 0.8	$1.3 \pm 0.5^{\#}$

The amplitudes of the tail currents induced by unconditioned depolarizations are expressed relative to that of conditioned tail current.
$^{\#}P < 0.05$ compared with the conditioned tail current.

In four cells, the set of three consecutive (unconditioned–conditioned–unconditioned) depolarizations, indicated by (a), (b) and (c) in Fig. 3, was applied six times. Changes in the amplitude and time constant of the tail currents evoked by this procedure are summarized in Table 2.

When 100 μM H-7, which inhibits a broad range of protein kinase activities (A-, G- and C-kinases, Hidaka et al., 1984), was applied to the perfusate (in four cells), the amplitude of the peak inward current, following depolarizing steps (0 mV, 75–100 ms, repeated at 30 s intervals), was significantly reduced to $82 \pm 7\%$ in 3 min. In the continuous presence of H-7, the same paired pulse protocol used in Fig. 2A was applied (conditioning depolarization to +80 mV for 5 s; test depolarization to 0 mV for 100 ms). Even in the presence of H-7, slow-tail current ($\tau_d = 9.2 \pm 1.7$ ms, $n = 7$) was induced by large conditioning depolarizations (Table 1B). On the other hand, the amplitude ($20.3 \pm 12.2\%$) and deactivation time constant ($\tau_d = 2.5 \pm 2.3$ ms) of the unconditioned tail currents were

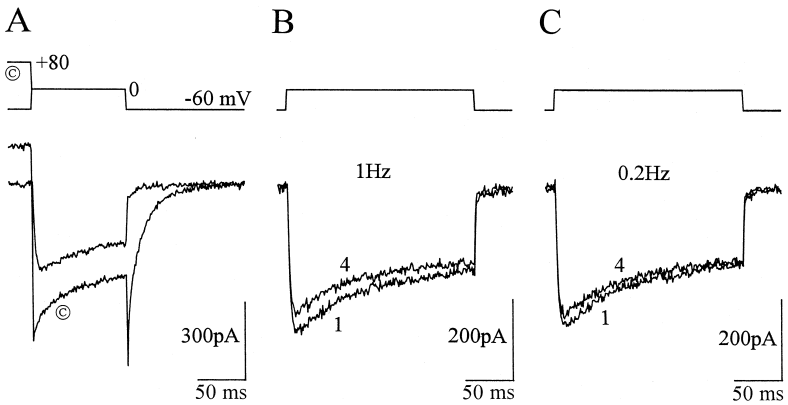

Fig. 4. Effects of repetitive depolarizations on Ca^{2+} channel current. After unconditioned and conditioned (conditioning step: +80 mV, 4 s; test step: 0 mV, 100 ms) Ca^{2+} currents had been obtained (A), four consecutive depolarizing pulses (0 mV, 200 ms) were applied at 1 (B) or 0.2 Hz (C). In (B) and (C), the membrane currents evoked by the first and fourth depolarizations are shown. The interval between the pulse sequences in (A)–(C) were approximately 45 s.

significantly smaller as compared to those of the unconditioned tail currents (Table 1B).

In one cell, in the presence of 1 μM forskolin, H-7 was added and the paired pulse protocol shown in Fig. 2A was applied. The results obtained were essentially the same as those obtained in the absence of forskolin.

3.4. Effects of GDP analogues

In N- and P/Q-type Ca^{2+} channels, facilitation caused by large preconditioning depolarization is known to involve a G-protein (Dolphin, 1998). Furthermore, in cardiac myocytes, L-type Ca^{2+} channel current is also modulated by G-proteins through changes in adenylate cyclase activity (Hescheler et al., 1986). Thus, we examined the possible involvement of G-protein in the development of the slow-tail current formation in guinea-pig detrusor cells. An amount of 1 mM GDP-β-S was added in the pipette solution. The paired pulse protocol shown in Fig. 2A was applied. The inclusion of GDP-β-S did not alter the conditions necessary to induce slow-tail currents: slow-tail currents were observed only after large conditioning depolarizations. The deactivation time constant of the tail current after large conditioning depolarization was 12.3 ± 3.0 ms ($n = 6$). On the other hand, the amplitude and time constant of the unconditioned tail current were significantly smaller than those of conditioned tail current (Table 1C).

When the extracellular Ca^{2+} was substituted with Ba^{2+}, qualitatively the same results were obtained in two cells.

3.5. Effects of frequency of depolarization

In cardiac muscle, it has been reported that potentiation of Ca^{2+} current induced by frequent depolarization is probably due to Ca^{2+} and cyclic AMP-dependent phosphorylation (Tiaho et al., 1994). After obtaining unconditioned and conditioned currents (conditioning step: +80 mV, 4 s; test step: 0 mV, 100 ms) (A), the effect of frequency of depolarization was examined (Fig. 4). In (B) and (C) the same depolarizing pulses (0 mV, 200 ms) were applied four times at 1 s and 5 s intervals, respectively. Unlike cardiac cells, four repetitive depolarizations at 1 and 0.2 Hz only reduced the amplitude of the peak inward current to 84 ± 5 and 92 ± 2 of the control, respectively ($n = 4$). Also, there was no slow-tail current observed in the fourth Ca^{2+} current.

Even when Ba^{2+} was used as a charge carrier, reduction of Ca^{2+} channel current was still observed. However, the degree of decrease in the current amplitude was less in Ba^{2+}-containing solution: the peak amplitude of the fourth inward current was decreased to $90.8 \pm 2.2\%$ at 1 Hz and $94.3 \pm 2.0\%$ at 0.2 Hz ($n = 6$).

4. Discussion

Previously, we have reported the presence of the multiple open states induced by combination of voltage steps in guinea-pig detrusor cells (Nakayama and Brading, 1993a, 1996). Large depolarizations transfer the conformation of smooth muscle L-type Ca^{2+} channels from the normal into a second open state (O_2) in which Ca^{2+} channels do not (or only very slowly) inactivate during depolarization, and upon repolarization, closure of Ca^{2+} channels is significantly slowed. As a result, slow deactivating Ca^{2+} tail currents of large amplitude are observed after large conditioning depolarizations. Slow-tail currents are also observed in the presence of Ca^{2+} channel agonists. However, the examination of the interaction of large depolarization and Ca^{2+} channel agonists suggested that the underlying mechanisms to produce slow-tail currents are distinct (Brading and Nakayama, 1993; Nakayama and Brading, 1995b): the time constants of the tail currents induced by large depolarization and Ca^{2+} channel agonists were dif-

ferent, and the combination of the two factors additively slowed the decay of the tail current.

Channel phosphorylation is known to modulate the Ca^{2+} current in cardiac and other cells (reviewed by Hess, 1990; McDonald et al., 1994). In the present study, we obtained several lines of evidence that the slow-tail current observed after large depolarization is not a result of high voltage-induced channel phosphorylation itself. In detrusor smooth muscle cells, isoprenaline slightly but consistently increased the amplitude of the Ca^{2+} current evoked by a simple depolarizing step (Fig. 1). However, unlike porcine coronary artery cells (Fukumitsu et al., 1990), in guinea-pig detrusor cells, forskolin, which directly activates adenylate cyclase, did not cause consistent potentiation of Ca^{2+} current, agreeing well with a previous report (Klöckner and Isenberg, 1985). Furthermore, when cAMP was included in the pipette, slow-tail currents were observed only after large conditioning depolarizations. These results suggest that the transition of Ca^{2+} channel conformation into the O_2 state is not due to A-kinase-dependent phosphorylation.

In smooth muscle, two mechanisms have been reported for isoprenaline-induced Ca^{2+} current potentiation which is not associated with cyclic AMP-dependent systems: (1) atypical adrenoceptors (C-type) in guinea-pig *Taenia caeci* (Muraki et al., 1993, 1994) and (2) G-protein associated mechanisms in bovine trachea (Welling et al., 1992) and in rabbit portal vein (Xiong et al., 1994). However, since the small and temporary increase in the Ca^{2+} current induced by isoprenaline did not seem to correlate with the slow-tail current formation in guinea-pig detrusor cells (no slow tail currents observed during isoprenaline application), further experiments were not performed to investigate the effect of isoprenaline.

In cardiac and neuronal L-type Ca^{2+} channels, it has been reported that voltage-dependent potentiation is due to phosphorylation through activation of protein kinase A (Sculptoreanu et al., 1993). On the other hand, in adrenal chromaffin cells, the potentiation of Ca^{2+} channel current has been explained by kinases other than A-kinase (Artalejo et al., 1992). In these experiments, the authors used a two-pulse protocol with an interpulse step, and demonstrated that H-7 reversibly inhibited potentiation of test inward current after preconditioning depolarizations (to $+120$ mV), and the large depolarization-induced potentiation of the test current was prolonged when ATP-γ-S was contained in the patch pipette. In contrast, in guinea-pig detrusor cells, the condition necessary to induce slow-tail current formation (large conditioning depolarization) is not affected by a high concentration of H-7 (Table 1B) or inclusion of ATP-γ-S in the patch pipette (Fig. 3). Thus, it seems unlikely that channel phosphorylation itself transfers the conformation of the Ca^{2+} channel from the normal (O_1) to a second open (O_2) state. This deduction is supported by the previous observation that slow-tail currents were induced by large depolarizations even though ATP was not included in the pipette (Nakayama and Brading, 1993a,b). However, the possibility that the O_1 to O_2 transition process (e.g., the rate, activation energy) is modulated by phosphorylation of channel protein still remains. Also, it is possible that some phosphorylation of the Ca^{2+} channel protein is required for basic channel activity and/or in shifting between gating modes (e.g., Ono and Fozzard, 1992; Groschner et al., 1996).

From the experiments investigating the interaction of large depolarization and Ca^{2+} channel agonist, as described above, it has been suggested that these factors operate separately and produce multiple open states: Ca^{2+} channel agonists convert the channel gating mode from mode 1 to mode 2, and each gating mode has two open states (O_1 and O_2 states in the gating mode 1 and O_1^* and O_2^* states in the mode 2; asterisks indicate Ca^{2+} channel agonist binding states). In both gating modes, the second open states (O_2 and O_2^*) are induced by large depolarization, but the deactivation of O_2^* is much slower than that of O_2 (Nakayama and Brading, 1995a). In the presence of a Ca^{2+} channel agonist, Bay K 8644, bath applications of 100 μM H-7 reduced the amplitude of the Ca^{2+} currents evoked by large conditioning depolarizations, but did not affect the time course of the tail current which decayed very slowly (unpublished observation). This result implies that even in the gating mode 2, the transition of Ca^{2+} channel conformation to the second open state (O_2^*) induced by the large conditioning depolarization does not correspond to channel phosphorylation process itself.

We also examined the possible contribution of Ca^{2+}- and cyclic AMP-dependent phosphorylation mechanism(s) underlying frequency-dependent facilitation of Ca^{2+} current seen in cardiac myocytes (Tiaho et al., 1994). In guinea-pig detrusor cells, however, high-frequency depolarization only reduced the amplitude of the Ca^{2+} current (Fig. 4), and it was also true when Ba^{2+} was used as a charge carrier.

It is suggested that N- and P/Q-type Ca^{2+} channel currents are suppressed by binding of G-protein to α1-subunit of these Ca^{2+} channels, and that voltage-dependent release of this protein restores the Ca^{2+} channel activity (reviewed by Dolphin, 1998). If formation of the slow-tail current involves an analogous mechanism, inclusion of GDP-β-S would affect the conditions necessary to induce slow-tail currents. However, when conditioned and unconditioned depolarizations (test steps) were alternately applied in the presence of GDP-β-S, slow tail currents were observed only after large preconditioning depolarizations: a reversible conditioning effect of large depolarization. This suggests that G-proteins are not coupled to the transition of Ca^{2+} channel conformation between the normal (O_1) and (O_2) open states.

Taken together, the O_1 to O_2 transition of the L-type Ca^{2+} channels during large depolarization seems not to depend on phosphorylation-like chemical reactions. It is suggested that the O_2 state is brought about by a novel

mechanism which L-type Ca^{2+} channel protein possesses internally, maybe by physical structural change of the Ca^{2+} channel during large depolarization.

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