

Interaction of Ca²⁺ Agonist and Depolarization on Ca²⁺ Channel Current in Guinea Pig Detrusor Cells

S. NAKAYAMA and A. F. BRADING

From the University Department of Pharmacology, Oxford, OX1 3QT, United Kingdom

ABSTRACT The interaction of large depolarization and dihydropyridine Ca²⁺ agonists, both of which are known to enhance L-type Ca²⁺ channel current, was examined using a conventional whole-cell clamp technique. In guinea pig detrusor cells, only L-type Ca²⁺ channels occur. A second open state (long open state: O₂) of the Ca²⁺ channels develops during large depolarization (at +80 mV, without Ca²⁺ agonists). This was judged from lack of inactivation of the Ca²⁺ channel current during the large depolarizing steps (5 s) and slowly deactivating inward tail currents (= 10–15 ms) upon repolarization of the cell membrane to the holding potential (–60 mV). Application of Bay K 8644 (in 2.4 mM Ca²⁺-containing solutions) increased the amplitude of the Ca²⁺ currents evoked by simple depolarizations, and made it possible to observe inward tail currents (= 2.5–5 ms at –60 mV). The open state induced by large depolarization (O₂*) in the Bay K 8644 also seemed hardly to inactivate. After preconditioning with large depolarizing steps, the decay time course of the inward tail currents upon repolarization to the holding potential (–60 mV) was significantly slowed, and could be fitted reasonably with two exponentials. The fast and slow time constants were 10 and 45 ms, respectively, after 2 s preconditioning depolarizations. Qualitatively the same results were obtained using Ba²⁺ as a charge carrier. Although the amplitudes of the inward currents observed in the test step and the subsequent repolarization to the holding potential were decreased in the same manner by additional application of nifedipine (in the presence of Bay K 8644), the very slow deactivation time course of the tail current was little changed. The additive enhancement by large depolarization and Ca²⁺ agonists of the inward tail current implies that two mechanisms separately induce long opening of the Ca²⁺ channels: i.e., that there are four open states.

INTRODUCTION

Ca²⁺ ions are involved in various intracellular processes. Modulators of Ca²⁺ channel behavior would have significant effects on a wide range of cellular functions: contraction, secretion, excitability (Hille, 1992; McDonald, Pelzer, Trautwein, and Pelzer, 1994). It is well known that dihydropyridine Ca²⁺ agonists, e.g., Bay K 8644, facilitate Ca²⁺ influx through voltage-dependent Ca²⁺ channels of excitable cells.

Address correspondence to A. F. Brading, University Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT, United Kingdom.

Such compounds induce numerous changes in the features of Ca^{2+} current kinetics: increase in peak amplitude and degree of inactivation, and hyperpolarizing shift of activation (cardiac myocytes: Sanguinetti, Krafte, and Kass, 1986; Markwardt and Nilius, 1988): slow deactivation of the tail current (Lacerda and Brown, 1989): voltage- and use-dependent modulation (Kamp, Sanguinetti, and Miller, 1989). In single-channel recording, an open state which has a slower closing time constant is observed (Hess, Lansman, and Tsien, 1984).

In smooth muscle, there are also many papers published on the effects of Ca^{2+} channel agonists (e.g., rabbit ear artery: Aaronson, Bolton, Lang, and MacKenzie, 1988; rat vena cava: Mironneau, Yamamoto, Sayet, Arnaudeau, Rakotoarisoa, and Mironneau, 1992; guinea pig taenia caeci: Lang and Paul, 1991; urinary bladder: Bonov and Isenberg, 1992; canine colon: Ward and Sanders, 1992). Although some discrepancies are seen in detailed modifications, in most smooth muscle Ca^{2+} channel currents are significantly potentiated by the agonists. Previously, in guinea pig detrusor cells, we reported an enhancement of Ca^{2+} current after large depolarization: a long open state (O_2) of the voltage-dependent Ca^{2+} channel, based on the observation that the inward tail current deactivated slowly ($= 10\text{--}15$ ms at -60 mV) after preconditioning by $+80$ mV steps (Nakayama and Brading, 1993*a*). Also, the Ca^{2+} channels in the long open state seemed to inactivate very slowly or not at all (Nakayama and Brading, 1993*b*). Similar phenomena have been reported in adrenal chromaffin cells (Hoshi and Smith, 1987) and in cardiac ventricular cells (Pietrobon and Hess, 1990). Since smooth muscles commonly show slow and/or tonic contractions, the underlying mechanism(s) for slow and sustained Ca^{2+} influx could play an important physiological role. Thus, it is of interest to examine the individual effects and interaction of large depolarization and Ca^{2+} agonists both of which prolong opening of the Ca^{2+} channels. In the present study, we performed such experiments, and found that the two factors and their combination induce three different tail currents.

METHODS

Preparation of Cells

The methods employed were essentially the same as previously described (Nakayama and Brading, 1993*a,b*). Male guinea pigs (450–740 g) were killed either by (*a*) stunning, (*b*) cervical dislocation and exsanguination, or (*c*) decapitation after anaesthesia by halothane. In case *c*, female guinea pigs were sometimes used. Single smooth muscle cells were enzymatically (0.05% collagenase, type 1 [Sigma Chemical Co., St. Louis, MO] and 0.1% pronase, [Fluka Chemical Co., Buchs, Switzerland]) dissociated from the urinary bladder. Some of the cell suspension was stored at 5°C before use, and used for up to 6 h.

Current Recording

The whole-cell membrane currents were recorded under voltage clamp mode (EPC 7, Germany) through a low-pass filter (3-pole, 10 kHz). The resistance of the patch pipette was in the region of 3–6 M Ω . Single smooth muscle cells of 40–80 pF membrane capacity were used for the electrical recordings. After rupture of the cell membrane, the series resistance was <10 M Ω . The capacitive surge was electrically compensated, the series resistance was partially compensated (by 50–70%).

The speed (time constant) of the clamp was set to be faster than 0.2 ms. The voltage error was normally <7 mV, especially when the time constant of the tail current was analyzed. Unless otherwise mentioned, the membrane potential was clamped at -60 mV (holding potential). An AD/DA converter (DT 2801A, Data Translation, United Kingdom) was used for voltage step generation and on-line data acquisition, supported by an IBM compatible computer. The computer programs were written with Quick Basic Software Package (Microsoft Corp.).

Solutions and Drugs

The normal bathing solution had the following composition (in millimolar): NaCl, 125; KCl, 5.9; CaCl₂, 2.5; MgCl₂, 1.2; glucose, 11.8 and HEPES (*N*-2-hydroxyethylpiperadine-*N*'-2-ethanesulphonic acid), 11.8; pH was adjusted to 7.4 with Tris base. In Ba²⁺-containing solution, 2.5 mM Ba²⁺ was added instead of Ca²⁺. To rule out contribution of external K⁺ to inward tail currents, in some experiments Ca²⁺ currents were recorded in Cs⁺-containing solution (instead of K⁺), and 7 mM TEA was added in the pipette solution. When Na⁺ currents through the Ca²⁺ channels (Fukushima and Hagiwara, 1985; Hess, Lansman, and Tsien, 1986) were measured, Ca²⁺ was removed and Mg²⁺ was reduced to 0.1 mM by isoosmotically substituting with Na⁺ and 1 mM EGTA (ethyleneglycol-bis-(β-aminoethylether) *N*, *N*, *N*', *N*'-tetraacetic acid) was added. K⁺ was also removed from this solution. The composition of the pipette solution was (in millimolar): CsCl, 141; MgCl₂, 1.4; EGTA, 2; ATP, 1; GTP (guanosine 5'-triphosphate), 0.1; HEPES/Tris, 10 (pH 7.2). All experiments were carried out at room temperature (24–28°C).

The following chemicals, drugs and enzymes were used in the present study: ATP (disodium salt), GTP (trisodium salt), EGTA, nifedipine, collagenase (type I) from Sigma Chemical Co.; Bay K 8644 from Calbiochem Corp. (La Jolla, CA); pronase from Fluka Chemical Co. Stock solutions of nifedipine and Bay K 8644 (dissolved with ethanol) were kept cool and protected from light. The drugs were diluted just before use.

Data Analysis and Statistics

The numerical data were expressed as means ± standard deviation (SD). The data used for statistics were obtained from cells initially showing a peak inward current of more than 400 pA at a step potential to 0 mV. When 100 nM Bay K 8644 was applied to the extracellular solution, the Ca²⁺ channel current repeatedly evoked by depolarization to 0 mV (100 ms, 30-s intervals) was increased in size just after the solution reached the recording cell, and a maximal effect was seen within 14 min. Subsequently, the Ca²⁺ channel current gradually declined during application (cell deterioration and/or run-down of the Ca²⁺ channels), but this rate varied considerably from cell to cell. Cells in which the Ca²⁺ channel current declined relatively rapidly were discarded.

Curve fitting of the decay of the inward currents was done by fitting the discrete data points iteratively with single ($N = 1$) or multiple ($N \geq 2$) exponential functions:

$$I(t) = A_0 + \sum_{k=1}^N A_k \exp(-t/\tau_k), \quad (1)$$

using a modified simplex program (Nakayama and Brading, 1993a). The total residual currents were used as a criterion for the convergence. At convergence, the mean residual currents were usually <8 pA or 2% of the peak inward current. Since we are mainly interested in slower tail currents than those normally seen after simple depolarizations, we set the digital resolution of the sampling to between 500 s and 2 ms. This sampling interval meant that the fast tail currents expected on repolarization from a simple depolarization were not resolved.

The relationship between the peak amplitude of Ca²⁺ current (I_{peakCa}) and voltage (E) of simple depolarizing step, was obtained using the following equations:

$$I_{\text{peakCa}}(E) = d_{\infty}(E) G_{\text{max}}(E - E_{\text{rev}}), \quad (2)$$

$$d_{\infty}(E) = 1 / (1 + \exp \{ (E_{0.5} - E) / S \}), \quad (3)$$

where G_{max} and E_{rev} are the maximum conductance and reversal potential of the current, respectively. $d_{\infty}(E)$ indicates voltage dependence of the degree of activation based on the Boltzmann distribution, where $E_{0.5}$ is the membrane potential at half maximal activation, and S is the slope factor.

RESULTS

Effects of Large Depolarization and Ca^{2+} Agonist

The individual effects and interaction of large depolarizations and Ca^{2+} channel agonists were compared, because both treatments cause long opening of the Ca^{2+} channels. The effects of Bay K 8644 were examined using conditioned (*B*) and unconditioned depolarizations (*A*) (Fig. 1). A test potential of +20 mV (100 ms) with

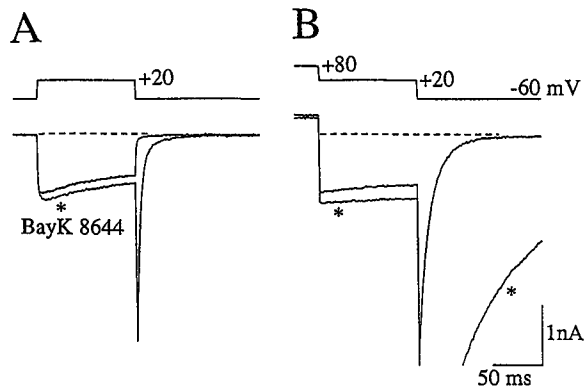


FIGURE 1. Effects of Bay K 8644 on the membrane currents evoked with (*B*) and without preconditioning (*A*). A test potential of +20 mV was repeated at 90-s intervals. A preconditioning step (+80 mV, 5 s) was applied before every other application of membrane depolarization. The last 25 ms is shown in *B*. The membrane currents observed 3 min after application of 100 nM Bay K 8644 are superimposed (indicated by *). In *B*, the tail current evoked in the presence of Bay K 8644 is only partially shown.

(*B*) or without preconditioning depolarization (+80 mV, 5 s) (*A*) were alternately applied at 90-s intervals. As previously described (Nakayama and Brading, 1993*b*), little inactivation of the Ca^{2+} channels occurs during +80 mV steps, and also depolarization at greater than +20 mV provides maximal activation. Thus, in normal solution, nearly the same peak amplitudes of the Ca^{2+} currents were observed during depolarization to +20 mV, irrespective of the preconditioning step. Application of Bay K 8644 (100 nM) similarly increased the amplitudes of the paired test inward currents evoked with or without preconditioning depolarization. In three cells, the averages of the increase in test current were 26% in both cases.

In normal solution, after conditioning depolarization at +80 mV, repolarization to the holding potential induced a tail current (Fig. 1 *B*) which had a decay time constant of 12.2 ms, as described previously (Nakayama and Brading, 1993*a*). In the presence of Bay K 8644, even when simple depolarization was applied (*A*)*, a large tail current was observed upon repolarization, but its time constant was shorter (4.4 ms). When the conditioning step was used in the presence of Bay K

8644, repolarization of the membrane to the holding potential induced a much larger tail current (which was unable to be fitted in this case, but the decay time constant normally ranged from 36–46 ms in the same condition: see Figs. 2–4). The enhancement induced by the large depolarization was reversible. Subsequent simple depolarization evoked a tail current having a time constant of 4 ms. Also, in different cells the decay time constant of the tail current induced after simple depolarization was not affected by changing the test potential to 0 mV (3.9 ± 0.8 ms after 0 mV; 3.7 ± 0.9 ms after +20 mV, $n = 5$). Similar results were produced in Ba²⁺-containing solutions.

The effect of Bay K 8644 on the relationship between the peak amplitude of Ca²⁺ current (I_{peakCa}) and the potential of the depolarizing step (E) was also examined. In normal solution (2.5 mM Ca²⁺) a series of depolarizing steps (-40 to +40 mV, 100 ms) were applied (at 30-s intervals). The I_{peakCa} maximized around 0 mV, and

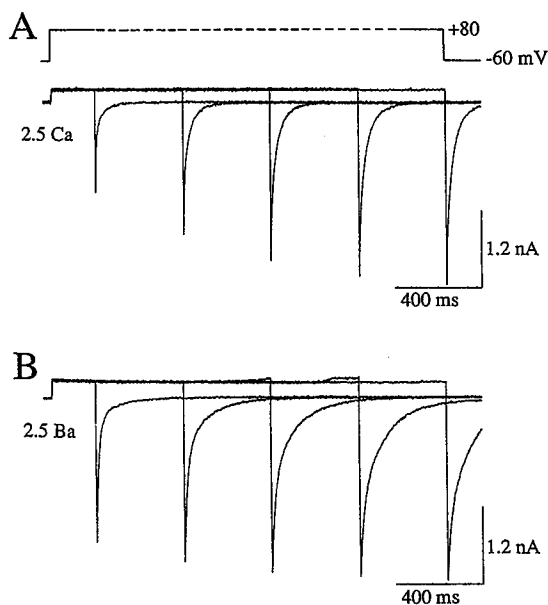


FIGURE 2. Effects of the duration of a large depolarization. In *A*, in the presence of 100 nM Bay K 8644 and 2.5 mM Ca²⁺, the duration of the +80 mV step was increased from 200 to 1,800 ms by 400-ms increments. The depolarizing steps were applied at 60-s intervals. In *B*, the same protocol was applied using 2.5 mM Ba²⁺ instead of Ca²⁺. Note that the relative amplitude of the slowly decaying component of the tail current was progressively increased by increasing the duration of the large depolarization.

then decreased as the positivity of the pulse increased. This $I_{\text{peakCa}}-E$ relationship was shifted in a hyperpolarizing direction by application of Bay K 8644 (100 nM). When the $I_{\text{peakCa}}-E$ curves were fitted with a linear maximum available Ca²⁺ current and Boltzmann's equation (Eqs. 2 and 3), the membrane potential at half maximal activation ($E_{0.5}$) appeared to shift from -14.5 ± 4.5 to -22.1 ± 4.3 mV ($n = 9$). Even when Ba²⁺ or Na⁺ was used as a charge carrier, similar hyperpolarizing shifts of the $I_{\text{peakCa}}-E$ relationship were observed (by -10 mV in Ba²⁺; by -11 mV in Na⁺).

Time-dependent Development of Slow Tail Current

Fig. 2 shows effects of the duration of the +80-mV step on the decay time course of the tail current evoked by returning the membrane potential to the holding potential (-60 mV). In *A*, in the presence of Bay K 8644 (100 nM) and 2.5 mM Ca²⁺, the

duration of the depolarizing step (+80 mV) was prolonged from 200 to 1,800 ms in 400-ms increments. Table I summarizes the development of the tail current observed in six cells. The amplitude of the tail current was progressively increased by increasing the duration of the depolarization at +80 mV, although the outward membrane current at the +80-mV step was little changed. The decay time course was fitted by the sum of two exponential terms. The fast decay time constant (fast) was 3.9 ± 0.7 ms after 200 ms depolarization, and progressively increased to 10.1 ± 2.8 ms after 1,400-ms depolarization ($n = 6$), while the slow decay time constant was relatively stable (ranging from 36 to 46 ms). The ratio of the amplitude of the slow/fast decay component was also increased by increasing the duration. In the presence of Ba^{2+} , the same tendency for the development of the slow decay component was seen, or rather more clearly demonstrated. The amplitude of the slow

TABLE I
Development of the Tail Current during Depolarization at +80 mV

Duration of +80 mV	200	600	1,000	1,400	1,800
	<i>ms</i>				
Peak amplitude	0.52 ± 0.07	0.73 ± 0.08	0.85 ± 0.05	0.94 ± 0.04	1
τ_{fast}	3.9 ± 0.7	5.9 ± 2.0	7.4 ± 2.8	10.1 ± 2.8	(-) ms
τ_{slow}	35.8 ± 2.2	40.2 ± 3.6	43.5 ± 5.9	46.2 ± 7.6	(-) ms
$A_{\text{fast}}/A_{\text{slow}}$	2.63 ± 0.99	1.14 ± 0.14	0.96 ± 0.29	0.86 ± 0.16	(-)

In the presence of 100 nM Bay K 8644 and 2.5 mM Ca^{2+} , the duration of the +80-mV step was changed. The measured peak amplitude of the tail current was normalized by that evoked after 1,800 ms depolarization. The decay time course of the tail current ($I_{\text{tail}}(t)$) was fitted by a sum of two exponential terms:

$$I_{\text{tail}}(t) = A_0 + A_{\text{fast}} \exp(t/\tau_{\text{fast}}) + A_{\text{slow}} \exp(t/\tau_{\text{slow}}).$$

The numerical data are expressed as means \pm SD ($n = 6$).

component was 14% after 200 ms, and 58% after 1,400-ms depolarization at +80 mV. Similar results were obtained from two more cells.

The time-dependent development of the slow component in the presence of Ba^{2+} suggests that this inward current is not due to Ca^{2+} -dependent conductances activated by the large tail current itself (e.g., Ca^{2+} -dependent nonselective cation channels (Inoue and Isenberg, 1990; Loirand, Pacaud, Baron, Mironneau, and Mironneau, 1990). Also, in the presence of 100 nM Bay K 8644 (normal solution) after 2-s depolarization at +80 mV, the cell membrane was transiently (5 ms) repolarized to -60 mV. Subsequently, the membrane was clamped at +80 mV again (see Fig. 3 in Nakayama and Brading, 1993a). The Cl^- concentrations of the pipette and bathing solutions were symmetric. If Cl^- conductances are activated upon repolarization, the amplitude after the repolarizing step should be larger. Since the membrane current at +80 mV was little affected by the preceding transient

repolarization (current trace not shown), it can be postulated that the tail currents do not involve a significant contribution of Cl⁻ conductance (e.g., Ca²⁺-activated Cl⁻ channels [Amédée, Large, and Wang, 1990]) or some other linear conductances. To rule out contribution of extracellular K⁺ to the inward tail current, most of the experimental procedures described above were repeated in Cs⁺-containing solutions (instead of K⁺). In these experiments, the pipette solution contained 7 mM TEA. Qualitatively similar results were reproduced. Taken together, the changes in decay time course evoked after large depolarization are considered to reflect altered Ca²⁺ channel kinetics.

Since the tail current evoked after large depolarization decays very slowly in the presence of dihydropyridine Ca²⁺ agonists, the voltage dependence of the maximum Ca²⁺ current (I_{\max} - V relationship) could be estimated. In the presence of Bay K 8644 (100 nM) and 2.5 mM Ca²⁺, 5-s depolarization to +80 mV was applied. Subsequently, the membrane potential was changed by various repolarizing steps (-60 to +20 mV). The voltage dependence of the maximum Ca²⁺ current is nearly linear as observed in the absence of Ca²⁺ channel agonists (Nakayama and Brading, 1993a).

Interaction of Dihydropyridine Ca²⁺ Agonists and Antagonists

Nifedipine, a dihydropyridine Ca²⁺ antagonist, is also known to act on L-type Ca²⁺ channels. The inhibitory effects of nifedipine were examined on the Ca²⁺ currents enhanced by Bay K 8644 and large depolarization. Fig. 3 A shows an example of an experiment. In the presence of 100 nM Bay K 8644, the same voltage sequence shown in Fig. 1 B (first step: +80 mV, 5 s; second step: 0 mV, 50 ms) was repeated at 90-s intervals (Fig. 3 A). Application of 1 μ M nifedipine gradually reduced the amplitude of both the inward currents (evoked at 0 and -60 mV), without affecting the outward current seen at +80 mV (B). The amplitudes fell to 13% of the control 6 min after application. Fig. 3 B shows the correlation between the peak amplitude of the inward current evoked at -60 mV (x -axis) and the amplitude at the end of the 0-mV step (y -axis). The amplitudes were normalized by the initial amplitudes for each dimension. This correlation plot also includes the data obtained by application of 1 μ M nifedipine in the presence of 1 μ M Bay K 8644 (*triangles*). The inhibitory ratio after 6 min was 51%. All data points lie very close to a line with a slope of unity, suggesting that L-type Ca²⁺ channels are responsible for all inward currents. However, the inhibitory effect of nifedipine seemed to be weaker than in the absence of Bay K 8644 as previously shown (half maximal inhibition was induced by 10~100 nM; Nakayama and Brading, 1993a). In another cell, in the presence of 100 nM Bay K 8644, application of 100 nM nifedipine reduced the inward currents only to 73% (data not shown). In Fig. 3 C, correlation of the amplitude (x -axis) and the decay time constant (τ_{slow} ; y -axis) of the tail current is shown. The decay time constant was stable during application of nifedipine, despite the reduction of the amplitude. A similar phenomenon was observed also in the absence Ca²⁺ agonists (Fig. 4; also seen in Fig. 4, Nakayama and Brading, 1993a). The tendency shown in the correlation plot C was essentially the same, even when the y -axis was the half decay time of the whole tail current (instead of τ_{slow}).

The interaction of Bay K 8644 and nifedipine on the Ca²⁺ channel currents was

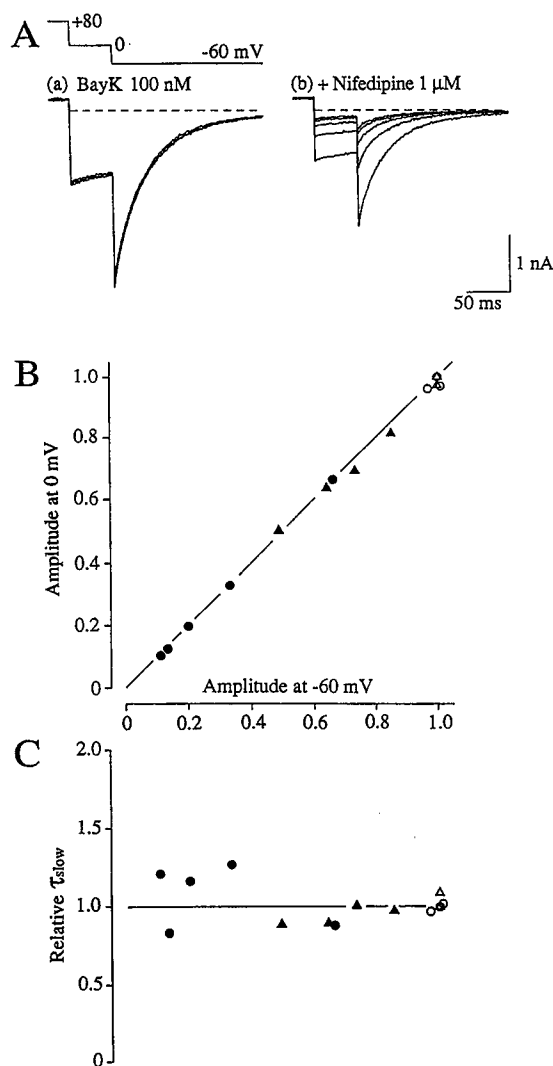


FIGURE 3. Inhibitory effects of nifedipine on the inward current evoked after large depolarization in the presence of Bay K 8644. In the presence of 100 nM Bay K 8644, the same voltage sequence shown in Fig. 1 *B* (first step: +80 mV, 5 s; second step: 0 mV, 50 ms) was repeated at 90-s intervals (*a*). Subsequently, 1 μ M nifedipine was added to the bathing solution (*b*). (*A*) The membrane currents observed during the experiment. (*B*) correlation between the peak amplitude of the tail current (-60 mV) and the current amplitude at the end (50 ms) of the 0-mV step. The currents were normalized by each control inward current initially obtained in the presence of only Bay K 8644. Open symbols correspond to the control data (*a*), while filled symbols are data in the additional presence of nifedipine. Circles are from the data shown in *A*. Triangles are from another experiment in which 1 μ M nifedipine was added in the presence of 1 μ M Bay K 8644. The solid line has a correlation coefficient = 1. In *C*, the slow decay time constant (τ_{slow}) is plotted against the peak amplitude of the tail current evoked by repolarization to -60 mV. The decay time course was iteratively fitted by the sum of two exponential

terms. The decay time constant was normalized by that initially obtained in the absence of nifedipine (control, *a*). The slow time constant was almost stable irrespective of the amplitude of the tail current. The solid line corresponds to the initial time constant. When the decay was fitted by a single exponential, a similar phenomenon was seen.

further investigated. In normal solution, without dihydropyridines, control membrane currents (Fig. 4 *A*) were obtained by the same voltage steps used in Fig. 3. Application of 100 nM nifedipine reduced the inward currents evoked at 0 and -60 mV (*B*) similarly to $\sim 10\%$ after 3 min. Subsequent addition of 0.5 μ M Bay K 8644 nearly fully restored the amplitude of the inward current evoked by the 0 mV step, while the tail current evoked at -60 mV became larger in size and slower in

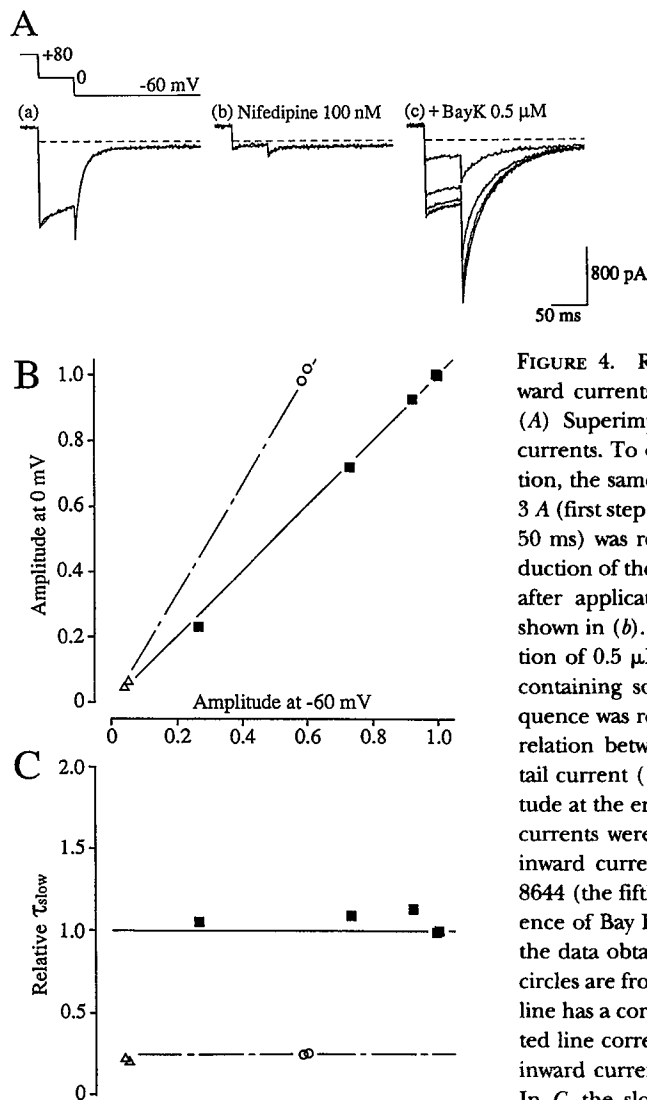


FIGURE 4. Recovery by Bay K 8644 of the inward currents in the presence of nifedipine. (A) Superimposed traces of the membrane currents. To obtain a control, in normal solution, the same voltage sequence shown in Fig. 3 A (first step: +80 mV, 5 s; second step: 0 mV, 50 ms) was repeated at 90-s intervals (a). Reduction of the inward currents observed 3 min after application of 100 nM nifedipine are shown in (b). Recovery brought about by addition of 0.5 μ M Bay K 8644 to the nifedipine-containing solution (c). In c, the voltage sequence was repeated at 60-s intervals. (B) correlation between the peak amplitude of the tail current (-60 mV) and the current amplitude at the end (50 ms) of the 0-mV step. The currents were normalized by each of the last inward currents after full recovery in Bay K 8644 (the fifth membrane current in the presence of Bay K 8644). Filled squares represent the data obtained from c. Open triangles and circles are from b and a, respectively. The solid line has a correlation coefficient = 1. The dotted line corresponds to the initial ratio of the inward currents measured at 0 and -60 mV. In C, the slow decay time constant (τ_{slow}) is plotted against the peak amplitude of the tail

current evoked by repolarization to -60 mV. The decay time constant of the tail current was normalized by that estimated from the last membrane current after application of Bay K 8644. The tail currents in the presence of Bay K 8644 were iteratively fitted by the sum of two exponential terms, while those in the absence of Bay K 8644 were fitted by a single exponential. The solid line corresponds to the time constant estimated from the last tail current (c). The dotted line corresponds to the initial time constant estimated from a.

decay time constant, compared to the control. Applications of either of the dihydropyridines had little effect on the membrane current during the +80-mV step. Although the ratio of the amplitudes of the inward currents at 0 and -60 mV was changed by application of Bay K 8644, during the recovery period both amplitudes

developed in the same manner (Fig. 4 *B*). Also, the decay time constants observed after application of Bay K 8644 were nearly the same (τ_{slow} ranged from 43 to 47 ms) (Fig. 4 *C*). A recovery having similar characteristics was observed when 200 nM Bay K 8644 was applied in the presence of 10 nM nifedipine. The results shown in Figs. 3 and 4 were reproduced in Ba^{2+} - or Cs^{+} -containing solutions, suggesting that the changes in tail currents reflected modulated Ca^{2+} channel behaviors by dihydropyridines.

DISCUSSION

In this paper, three types of large tail currents have been shown by combinations of Ca^{2+} agonists and depolarizing steps. Firstly, in normal solution (containing 2.5 mM Ca^{2+}), after large conditioning depolarization (+80 mV), repolarization of the membrane to the holding potential evoked a large tail current which had a decay time constant of 10 ~ 15 ms (Fig. 3 *B*). In a previous paper (Nakayama and Brading, 1993*a*), this tail current has already been identified as a Ca^{2+} current flowing through the same L-type Ca^{2+} channels that are responsible for the inward currents normally observed during simple depolarizing steps, but the channels are in a long opening configuration induced by the large depolarization.

The other two tail currents were observed in the presence of Bay K 8644, a typical Ca^{2+} channel agonist. This compound is known to act on L-type Ca^{2+} channels and induce long opening (e.g., Hess et al., 1984; Hess, 1990). In Ca^{2+} -containing solution, even after simple depolarization, repolarization of the membrane to the holding potential induced a large tail current in the presence of Bay K 8644 (Fig. 1 *A**). This tail current had a decay time constant of 35 ms. Similarly, when Ba^{2+} or Na^{+} was used as a charge carrier, Bay K 8644 induced tail currents upon repolarization from simple depolarizations. These results suggest that the tail currents observed in the presence of Ca^{2+} agonists are due to the Ca^{2+} channels (L-type), being consistent with previous observations (e.g., Lacerda and Brown, 1989; Yue, Herzig, and Marban, 1990).

When large depolarization (+80 mV) was applied as a preconditioning step in the presence of Bay K 8644, repolarization of the membrane to the holding potential induced a much larger tail current than those described above. Several lines of evidence suggest that this large tail current is also due to L-type Ca^{2+} channels: the almost linear voltage dependence of I_{max} (see also Fig. 9 in Nakayama and Brading, 1993*a*); the fact that nifedipine reduced the inward currents evoked by the test potential (0 mV) and repolarization to the holding potential, after large conditioning depolarization, in the same manner (Fig. 3); also, reduction of inward current induced by nifedipine was reversed by Bay K 8644 (Fig. 4), being consistent with the antagonism between these dihydropyridine derivatives in L-type Ca^{2+} channels (e.g., Spedding and Berg, 1984; Schramm and Towart, 1984; Brown, Kunze, and Yatani, 1986).

Thus, the three tail currents can be attributed to modulation of the same L-type Ca^{2+} channels by Ca^{2+} agonists and large depolarizing steps. Modulation of the behaviors of L-type Ca^{2+} channels by Ca^{2+} channel agonists and antagonists, is usually explained by the mode gating theory (Hess et al., 1984): mode 0 (null mode, un-

available for opening) favored by Ca²⁺ channel antagonists; mode 1 (brief opening); mode 2 (long-lasting channel opening) favored by Ca²⁺ channel agonists. In guinea-pig detrusor cells, we have previously proposed a large depolarization-mediated long open state (O₂): C-O₁-O₂, where O₁ is normal open state (Nakayama and Brading, 1993a). Since the large conditioning depolarization and Ca²⁺ agonists additively affected the formation of the inward tail current, we can postulate that the two factors separately induce long channel opening. Thus, mode 1 and mode 2 could be rewritten as C-O₁-O₂ and C*-O₁*-O₂* (asterisks indicate states with Ca²⁺ agonist bound; two open states in each mode), leaving out inactivation mechanisms. The present results can be explained by a simple combination of the two kinetic schemes described above, although many other complex models are also possible. For example, the mode 1 and mode 2 may be divided into submodes: mode 1 (C-O₁ and C-O₂); mode 2 (C*-O₁* and C*-O₂*). Detailed kinetics of the Ca²⁺ channels may be elucidated by further investigation, but in the present study we have used the simplest kinetic scheme to explain our results. The decay time constant of the tail current evoked after simple depolarization in the presence of Bay K 8644 would correspond to the O₁* to C* transition, while that seen after large conditioning step in the absence of Ca²⁺ agonists corresponds to the O₂ to O₁ transition. Also, the O₂* state would correspond to the slowest deactivation induced by a combination of large depolarization and Bay K 8644 ($\tau_{\text{slow}} \approx 36\text{--}46$ ms).

Although nifedipine reduced the amplitude of the tail current in the presence of Bay K 8644, the time constant of the tail current was not reduced (Fig. 3). This phenomenon could be explained by invoking a null mode (mode 0): some population of the Ca²⁺ channels are transformed to this unavailable for opening state, and the rest of the channels deactivate through the transition of O₂* to O₁*. Conversely, it could be postulated that addition of Bay K 8644 in the presence of nifedipine increases the population of Ca²⁺ channels in the mode favored by Ca²⁺ agonists (mode 2). It may be noteworthy that after application of Bay K 8644, the tail currents (at -60 mV) became larger in size than the control (Fig. 4 A). The excess recovery of the tail current could be due to a slower transition of O₂* to O₁* configuration (compared to that of O₂ to O₁) during the test step (0 mV, 50 ms).

The amplitude of the macroscopic current (I_{Ca}) can be expressed by introducing the availability of the Ca²⁺ channels (P_s):

$$I_{\text{Ca}} = NP_s P_o i_{\text{Ca}}, \quad (4)$$

where N and i_{Ca} are the number and unit current amplitude of the Ca²⁺ channels, respectively, and P_o is the degree of activation [$d_{\infty}(E)$ in Eq. 2] (Ochi, 1993). In bovine coronary artery smooth muscle cells, Isenberg and Klöckner (1985) have reported that neither Bay K 8644 (100 nM) nor nifedipine (1 μM) affected the unitary conductance of the Ca²⁺ channel. Since maximal activation is achieved at +20 mV, the increase in the test current by Bay K 8644 shown in Fig. 1 could be attributed to changes in the availability (P_s) of the Ca²⁺ channels. However, in the present experiments, we cannot rule out the possibility of an increase in unit conductance of Ca²⁺ channels in the presence of Bay K 8644, as has been suggested by Lacerda and Brown (1989). Also other mechanisms, which operate separately from the transitions shown in the scheme, may be affected by Ca²⁺ agonists (e.g., the fast

voltage-independent C-O transition which is responsible for preventing P_o reaching unity even at extreme depolarizing voltages).

The tail currents induced by either preconditioning depolarization at +80 mV (5 s) (e.g., Fig. 1 *B*, the current trace without asterisk indication) or simple depolarization with Bay K 8644 (e.g., Fig. 1 *A**) could be fitted with a single exponential function. From the results presented in this study, we postulated the presence of four open states: O_1 , O_2 , O_1^* , and O_2^* . Although the tail currents in both of these cases will involve the O_1 state, the single exponential fit of the tail currents mainly reflects deactivation from either the O_2 or O_1^* state. The O_1 to C transition is significantly faster than the digital resolution (0.5–2 ms) used in the present study, and therefore Ca^{2+} tail currents due to this transition are not resolved. Also, incomplete cancellation of the capacitive surge and the speed of the clamp, affect the analyses of the decay time constants of the tail currents. Tail currents induced after a preconditioning large depolarization in the presence of Bay K 8644 (e.g., Fig. 1 *B**), in contrast to the other tail currents were fitted best with a double exponential function. Using three or more exponential terms did not give reasonable convergence. As the deactivating time constants of O_2 (8 or 10–15 ms) and O_1^* (3–5 ms) are relatively similar (compared to that of O_2^* : 30–50 ms), the tail currents induced by large depolarization in the presence of Bay K 8644 can be fitted with two exponential terms. The increase in τ_{fast} shown in Table I can be explained by a relative increase in O_2 (compared to O_1^*) during large depolarizations. The fitting of the tail current is, of course, further complicated by the factors already discussed. However, our analyses of the tail currents were able to qualitatively distinguish the deactivating time constants of the extra three open states: O_2 , O_1^* , and O_2^* .

Off gating currents will be included in the tail current analyses. The reported time constant of the gating current (0.2–0.5 ms: Ertel, Smith, Leibowitz, and Cohen, 1994) is too fast to be followed in our experiments. However, the decay of the gating current may be slowed after large depolarization. In further experiments using either Cadmium or toxins which block Ca^{2+} current, e.g., Ω -Aga-IIIa, we may be able to quantify the contribution of these slowed gating currents to the slower tail currents which we are able to resolve.

Is there any evidence that these long channel open states play any physiological role? In cardiac cells, it has been reported that a channel opening similar to mode 2 gating is induced by high concentrations of β -adrenergic agonists (Yue et al., 1990) and is probably due to the complete phosphorylation of the Ca^{2+} channel protein (Ono and Fozzard, 1993). Furthermore, it has recently been shown that potentiation (increase in size of the peak amplitude and slower inactivation) of Ca^{2+} current induced by repetitive depolarization (e.g., Noble and Shimoni, 1981; Mitra and Morad, 1986; Richard, Charnet, and Nerbonne, 1993) is linked to phosphorylation processes, perhaps Ca^{2+} and cyclic AMP-dependent phosphorylation (Tiaho, Piot, Nargeot, and Richard, 1994). These facts have opened a possibility that mode 2-like gating mechanisms may occur even under physiological conditions. On the other hand, large depolarizing steps, under experimental conditions, may induce a full transition to a different type of long open state. This mechanism probably becomes effective in some of the Ca^{2+} channels during the larger agonist-induced depolarizations occurring in response to parasympathetic nerve stimula-

tion, and long channel openings may thus play a role in the tonic contraction required during micturition. Also, differences in the availability of multiple open states of the Ca²⁺ channels may be important in determining the varieties of contractile behavior which are a feature of smooth muscles.

In conclusion, the three types of the tail currents induced by combinations of dihydropyridine Ca²⁺ agonists and depolarizing steps can all be attributed to the same origin (L-type Ca²⁺ channels). The decay time constant of the tail current observed after simple depolarization in the presence of Ca²⁺ agonists differs from that induced by large conditioning depolarizations (+80 mV) before application of Ca²⁺ agonists. Furthermore, these two factors (Ca²⁺ agonist and large depolarization) additively slowed the decay time course, suggesting that the underlying mechanisms operate separately and that under these conditions there are four possible open states.

The authors are grateful to Dr. Derek Terrar for pertinent help and discussion.

This work was carried out during the tenure of a grant from Bristol Myers Squibb.

Original version received 17 October 1994 and accepted version received 16 May 1995.

REFERENCES

- Aaronson, P. I., T. B. Bolton, R. J. Lang, and I. MacKenzie. 1988. Calcium currents in single isolated smooth muscle cells from the rabbit ear artery in normal-calcium and high-barium solutions. *Journal of Physiology*. 405:57-75.
- Amédée, T., W. A. Large, and Q. Wang. 1990. Characteristics of chloride currents activated by noradrenaline in rabbit ear artery cells. *Journal of Physiology*. 428:501-516.
- Bonov, A., and G. Isenberg. 1992. Arginine-vasopressin induces mode-2 gating in L-type Ca²⁺ channels (smooth muscle cells of the urinary bladder of the guinea-pig). *Pflügers Archiv*. 420:219-222.
- Brown, A. M., D. L. Kunze, and A. Yatani. 1986. Dual effects of dihydropyridines on whole cell and unitary calcium currents in single ventricular cells of guinea-pig. *Journal of Physiology*. 379:495-514.
- Ertel, E. A., M. M. Smith, M. D. Leibowitz, and C. J. Cohen. 1994. Isolation of myocardial L-type calcium channel gating currents with the spider toxin Ω -Aga-IIIa. *Journal of General Physiology*. 103:731-753.
- Fukushima, Y., and S. Hagiwara. 1985. Current carried by monovalent cations through calcium channels in mouse neoplastic B lymphocytes. *Journal of Physiology*. 358:255-284.
- Hess, P. 1990. Calcium channels in vertebrate cells. *Annual Review of Neuroscience*. 13:337-356.
- Hess, P., J. B. Lansman, and R. W. Tsien. 1984. Different modes of Ca channel gating behaviour favored by dihydropyridine Ca agonists and antagonists. *Nature*. 311:538-544.
- Hess, P., J. B. Lansman, and R. W. Tsien. 1986. Calcium channel selectivity for divalent and monovalent cations: voltage and concentration dependence of single channel current in ventricular heart cells. *Journal of General Physiology*. 88:293-319.
- Hille, B. 1992. Calcium channels. In *Ionic Channels of Excitable Membranes*. Sinauer Associates Inc., Sunderland, MA. 83-114.
- Hoshi, T., and S. J. Smith. 1987. Large depolarization induces long openings of voltage-dependent calcium channels in adrenal chromaffin cells. *Journal of Neuroscience*. 7:571-580.
- Inoue, R., and G. Isenberg. 1990. Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. *Journal of Physiology*. 424:73-92.
- Isenberg, G., and U. Klöckner. 1985. Elementary currents through single Ca channels in smooth muscle cells isolated from bovine coronary arteries. Effects of nifedipine and Bay K 8644. *Pflügers*

Archiv. 403:R23.

- Kamp, T. J., M. C. Sanguinetti, and R. J. Miller. 1989. Voltage- and use-dependent modulation of cardiac calcium channels by the dihydropyridine (+)-202-791. *Circulation Research*. 64:338-351.
- Lacerda, A. E., and A. M. Brown. 1989. Nonmodal gating of cardiac calcium channels as revealed by dihydropyridines. *Journal of General Physiology*. 93:1243-1273.
- Lang, R. J., and R. J. Paul. 1991. Effects of 2,3-butanedione monoxime on whole-cell Ca^{2+} channel currents in single cells of the guinea-pig taenia caeci. *Journal of Physiology*. 433:1-24.
- Loirand, G., P. Pacaud, A. Baron, C. Mironneau, and J. Mironneau. 1991. Large conductance calcium-activated non-selective cation channel in smooth muscle cells isolated from rat portal vein. *Journal of Physiology*. 437:461-475.
- Markwardt, F., and B. Nilius. 1988. Modulation of calcium channel currents in guinea-pig single ventricular heart cells by the dihydropyridine BAY K 8644. *Journal of Physiology*. 399:559-575.
- McDonald, T. F., S. Pelzer, W. Trautwein, and D. Pelzer. 1994. Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiological Reviews*. 74:365-507.
- Mironneau, J., T. Yamamoto, I. Sayet, S. Arnaudeau, L. Rakotoarisoa, and C. Mironneau. 1992. Effect of dihydropyridines on calcium channels in isolated smooth muscle cells from rat vena cava. *British Journal of Pharmacology*. 105:321-328.
- Mitra, R., and M. Morad. 1986. Two types of calcium channels in guinea-pig ventricular myocytes. *Proceedings of the National Academy of Sciences of the USA*. 83:5340-5344.
- Nakayama, S., and A. F. Brading. 1993a. Evidence for multiple open states of the Ca^{2+} channels in smooth muscle cells isolated from the guinea-pig detrusor. *Journal of Physiology*. 471:87-105.
- Nakayama, S., and A. F. Brading. 1993b. Inactivation of the voltage-dependent Ca^{2+} channel current in smooth muscle cells isolated from the guinea-pig detrusor. *Journal of Physiology*. 471:107-127.
- Noble, S., and Y. Shimoni. 1981. The calcium and frequency dependence of the slow inward current 'staircase' in frog atrium. *Journal of Physiology*. 310:57-75.
- Ochi, R. 1993. Single-channel mechanism of β -adrenergic enhancement of cardiac L-type calcium current. *Japanese Journal of Physiology*. 43:571-589.
- Ono, K., and H. A. Fozzard. 1993. Two phosphatase sites on the Ca channel affecting different kinetic functions. *Journal of Physiology*. 470:73-84.
- Pietrobon, D., and P. Hess. 1990. Novel mechanism of voltage-dependent gating in L-type calcium channels. *Nature*. 346:651-655.
- Richard, S., P. Charnet, and J. N. Nerbonne. 1993. Interconversion between distinct gating pathways of the high threshold calcium channel in rat ventricular myocytes. *Journal of Physiology*. 462:197-228.
- Sanguinetti, M. C., D. S. Krafte, and R. S. Kass. 1986. Voltage-dependent modulation of Ca channel current in heart cells by Bay K 8644. *Journal of General Physiology*. 88:369-392.
- Schramm, M., and R. Towart. 1984. Modulation of calcium channel function with 1,4-dihydropyridine structure. *Journal of Physiology*. 349:53P. (Abstr.)
- Spedding, M., and C. Berg. 1984. Interactions between a "calcium channel agonist," Bay K 8644, and calcium antagonists differentiate calcium antagonist subgroups in K^+ -depolarized smooth muscle. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 328:69-75.
- Tiaho, F., C. Piot, J. Nargeot, and S. Richard. 1994. Regulation of the frequency-dependent facilitation of L-type Ca^{2+} currents in rat ventricular myocytes. *Journal of Physiology*. 477:237-252.
- Ward, S. M., and K. M. Sanders. 1992. Upstroke component of electrical slow waves in canine colonic smooth muscle due to nifedipine-resistant calcium current. *Journal of Physiology*. 455:321-337.
- Yue, D. T., S. Herzog, and E. Marban. 1990. β -adrenergic stimulation of calcium channels occurs by potentiation of high-activity gating modes. *Proceedings of the National Academy of Sciences, USA*. 87:753-757.