Cyclic-AMP-dependent phosphorylation modulates the stereospecific activation of cardiac Ca channels by Bay K 8644*


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Abstract. Voltage-gated Ca channels have been reported to be regulated by membrane potential, phosphorylation and binding of specific agonists or antagonists such as dihydropyridines. We report here evidence that cyclic AMP (cAMP) modulates the activation of Ca-channel current by the dihydropyridine agonist Bay K 8644. Bay K 8644 (racemate) alone induces a primary voltage-dependent, potentiating effect on peak current amplitude and accelerates the current decay. In contrast, in the presence of cAMP activators, we observed a striking slowing of the decay in addition to the increase in peak current. The agonist (-)-Bay K 8644, but not the antagonist (+)-Bay K 8644, when applied in combination with cAMP, forskolin or isoproterenol, mimics the effect of the racemate. We have interpreted the results presented here in respect of a cAMP-dependent modulation of Bay K 8644 effects on cardiac Ca-channel currents. It may open the new perspective that dephosphorylated and phosphorylated Ca channels have distinct pharmacology.

Key words: Ca channel current – Dihydropyridine agonist Bay K 8644 – cAMP-dependent modulation – Stereospecific interaction

Introduction

Since their opening is steeply voltage-dependent, dihydropyridine-sensitive L-type Ca channels are prime targets for modulation of cardiac function by neurotransmitters and direct agonists or antagonists such as dihydropyridines. It is well documented that β-adrenergic receptor activation increases the amplitude of Ca currents (I_{Ca}) in myocardial tissues by a mechanism involving stimulation of adenylate cyclase, increased production of cAMP and activation of cAMP-dependent protein kinase [for review, see Reuter (1983) and Levitan (1988)]. β-Adrenergic agonists increase the number and the opening probability of functional Ca channels (Cachelin et al. 1983; Reuter et al. 1983; Brum et al. 1984; Tsien et al. 1986), suggesting that protein phosphorylation leads to an increase in channel availability (Kameyama et al. 1986). In addition, the stimulatory guanosine-nucleotide-binding (G) protein also appears to exert a direct effect on Ca channels (Yatani et al. 1987), supplementary to its indirect activation through the cAMP cascade.

Although Bay K 8644 and β-adrenergic agonists have in common a positive inotropism, the effects observed at the single-channel level are fundamentally different (Kokubun and Reuter 1984; Tsien et al. 1986). Maximally effective doses of Bay K 8644 do not occlude the positive inotropic effect of isoproterenol (Thomas et al. 1985) suggesting distinct mechanisms of action. For example, Bay K 8644: (a) does not increase cAMP levels in the cells (Kokubun and Reuter 1984); (b) displays both agonist or antagonist activity depending on membrane voltage (Sanguinetti et al. 1986) and on the optical enantiomer used (Kass 1987); and (c) promotes a pattern of gating typified by prolonged mean open time (Hess et al. 1984; Kokubun and Reuter 1984; Ochi et al. 1984) and/or an increase in the probability of reopening of a single Ca channel (Brown et al. 1984).

In light of these data, an interesting question can be posed: how would Bay K 8644 and β-adrenergic agents act, when applied in combination, on I_{Ca}? We report here that, in addition to the cumulative effect on peak current amplitude, application of isoproterenol and pure agonist (-)-Bay K 8644 in combination markedly slows the inactivation and deactivation kinetics of Ca currents in cardiac myocytes. This latter effect is strikingly distinct from the acceleration induced by each drug employed alone.

Materials and methods

Ca-channel current isolation. Experiments were carried out on single ventricular myocytes obtained by collagenase dissociation (Wittenberg...
from 6- to 10-week-old rats. Whole-cell recordings of Ca current ($I_{Ca}$) and Ba current ($I_{Ba}$) were obtained at 20–22°C under conditions optimized to eliminate contaminating voltage-gated inward Na and outward K currents (Hess et al. 1986; Hadley and Hume 1988; Kass 1987; Richard et al. 1989, 1990). Bath solutions contained (in mM): NaCl (or NaCH3COO) 140, CaCl2 (or BaCl2) 2, MgCl2 2, HEPES 10, glucose 10 at pH 7.2 (NEt4OH or NaOH). 20 μM tetrodotoxin was added to block most of the Na channels. NEt4Cl (tetraethylammonium chloride) was used instead of NaCl, in order to ensure complete suppression of tetrodotoxin-resistant Na channels when studying $I_{Ca}$ at a holding potential less than ~50 mV. Non-specific Na currents ($I_{Na,n}$) flowing through Ca channels were also recorded in the absence of divalent cations (presence of 1 mM EDTA). Recording pipettes contained (in mM): CsCl 140, EGTA 10, HEPES 10, (Mg)ATP 3, (Mg)GTP 0.4, glucose 10 at pH 7.2 (CsOH), unless otherwise noted.

**Electrophysiological recordings.** The whole-cell variation of the patch-clamp technique (Hamill et al. 1981) was employed using a Biologic (model RK300) patch-clamp amplifier. Recording pipettes were fire-polished prior to use (tip diameters: 1–2 μm; resistances: 2–4 MΩ) when filled with recording solutions. After formation of a tight seal between the recording electrode and the myocyte membrane, electrode capacitance was compensated electronically prior to obtaining a whole-cell recording (seals resistance > 1 GΩ). After membrane disruption, series resistances (estimated from the decay of the capacitive transients) were measured using a ±10-mV test pulse (sampling frequency 50 kHz). Mean values were 6.7 ± 1.0 MΩ and 1.4 ± 0.3 MΩ (n = 14) respectively, before and after electronic compensation (79.2 ± 2.3%). Since all measured current amplitudes were below 4 nA, we estimate the maximal voltage errors resulting from the uncompensated series resistance less than 7 mV. The remaining series resistances are given for all original records. All experimental parameters, such as holding potentials, test potentials etc., were controlled with an IBM PC equipped with a Teeman Labmaster analog interface (Axon instruments) to the electrophysiological equipment. Sampling frequencies ranged from 1 kHz to 50 kHz. Current signals were filtered at 3 kHz. Data acquisition and analyses were performed using the pCLAMP software (Axon instruments). Prior to and during drug applications, Ca-channel current was routinely measured during (125 ms duration) depolarizations to potential evoking nearly maximal peak current activation (~10 mV for $I_{Ca}$; 0 mV for $I_{Ca}$) from a holding potential ~80 mV at 8- to 15-s intervals. The $I_{Ca}$ peak was measured as the difference between the maximal inward current amplitude and the zero current level ($I_{0}$, dashed lines in the figures). We have obtained no evidence for non-inactivating components of $I_{Ca}$ or for T-type currents in these cells (Richard et al. 1989, 1990). The time of half inactivation ($t_{1/2}$) was measured between $I_{Ca}$ peak current and $I_{0}$. Results are expressed as means ± SD. Statistical analysis was made using a binomial test based on the sign of the difference between paired samples (Table 1).

**Drugs.** Bay K 8644 [racemate, (−) and (+) enantiomers] and nicardipine were kindly supplied by Dr. Traber (Bayer AG) and Sandoz laboratory, respectively. Bay K 8644 and nicardipine were dissolved in 50% ethanol to make a concentrated stock solution (10 mM), which was stored at ~20°C. Isoproterenol (Sigma) was dissolved in H2O (10 mM stock solution) as required. Test solutions were prepared daily using aliquots from frozen stores to obtain the working concentrations. Controls revealed that the solvent has no effects on Ca-channel currents at the final dilutions used here (always < 0.005%). Solutions were applied to the exterior of the cell using a perfusion system (200 μm inner diameter capillary tubing flowing at a rate of 0.5 ml/min) placed in the vicinity of the cell (<1 mm) in a 2-ml bath chamber.

**Results**

**Effects of Bay K 8644 and isoproterenol on Ca-channel currents**

Exposure to the Ca$^{2+}$ agonist Bay K 8644 (racemic mixture) increased Ca current ($I_{Ca}$) in rat ventricular cells (Fig. 1A). Under the recording conditions employed here (holding potential of ~80 mV), we observed a maximal increase (2.9 ± 0.5 times, n = 10) at 1 μM. Current-activation and -inactivation curves were shifted in the hyperpolarizing direction [data not shown but c.f. Sanguinetti et al. (1986) and Kass (1987)]. In addition, the apparent rate of $I_{Ca}$ decay was often accelerated as exemplified by comparison of the times of half-inactivation ($t_{1/2}$) in Fig. 1A. Although this effect confirms previous observations on $I_{Ca}$ (Brown et al. 1984; Cohen and Lederer 1987; Hadley and Hume 1988; Lacerda and Brown 1989), under our standard experimental conditions (see Materials and methods) the acceleration was observed on only 80% of the cells tested (n = 10). In contrast, a slowing was observed in the 20% remaining cells. However, we found that the acceleration occurred on all cells (n = 13) when exogenous ATP was removed from the pipette solution (data not shown). When Ba$^{2+}$ ions were used as charge carriers in standard conditions, the acceleration of $I_{Ba}$ decay occurred on all cells (see Table 1) and this effect was markedly better than that observed with Ca$^{2+}$ (Fig. 1B), as would be anticipated from previous work (Hess et al. 1984; Sanguinetti et al. 1986; Markwardt and Nilius 1988). The same marked acceleration was observed with Na$^{+}$ flowing through the channels ($I_{Na,not}$ data not shown).

A measurable alteration of the deactivating inward tail currents could be observed after the addition of Bay K 8644, which was better demonstrated on $I_{Ba}$ (Fig. 1B). This effect