Modulation by Stimulation Rate of Basal and cAMP-elevated Ca\(^{2+}\) Channel Current in Guinea Pig Ventricular Cardiomyocytes

**SARKIS P. KASPAR and DIETER J. PELZER*\(^{1}\)**

From the Membrane Transport and Signaling Group, Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4H7

**ABSTRACT** The modulation of L-type Ca\(^{2+}\) current (\(I_{Ca}\)) by changes in stimulation frequency was investigated in single ventricular cardiomyocytes isolated from guinea pig hearts. Electrical recordings were carried out at 21–25°C and at 33–37°C with the whole-cell patch clamp method, under K\(^+\)-free conditions. A comparison is made between the response to frequency changes for \(I_{Ca}\) in the basal state and after the application of drugs which elevate the level of adenosine-3',5'-cyclic monophosphate (cAMP) within the cells. Peak basal \(I_{Ca}\) was reduced with an increase in stimulation rate from 0.5 Hz to 1, 2, 3, 4, or 5 Hz. This frequency-induced reduction of \(I_{Ca}\) was enhanced by reduced temperature, was unchanged when Na\(^+\) or Ba\(^{2+}\) carried the basal Ca\(^{2+}\) channel current, and was greatly enhanced after elevating cAMP levels with forskolin, isoprenaline, or 8-(4-chlorophenylthio)-cyclic AMP. We examined the mechanism of the enhancement of the frequency-induced reduction of \(I_{Ca}\) by cAMP, and found two conditions which abolished it: (a) application of isoprenaline when Na\(^+\) carried the Ca\(^{2+}\) channel current in Ca\(^{2+}\)-free solution, or (b) application of 3-isobutyl-1-methylxanthine, a broad-spectrum phosphodiesterase inhibitor. It was further shown that an elevation of both \(I_{Ca}\) and cAMP (induced by isoprenaline), and not an increase of \(I_{Ca}\) alone (induced by Bay K 8644), is required to produce the extra component of reduction by frequency. It is concluded that Ca\(^{2+}\) entry results in feedback regulation of \(I_{Ca}\), through the activation of Ca\(^{2+}\)-dependent phosphodiesterase(s). This is important in the context of sympathetic stimulation, which produces the companion conditions of an elevated heart rate and increases in cAMP levels and Ca\(^{2+}\) entry.

**INTRODUCTION**

Increases in stimulation rate elevate the intracellular Ca\(^{2+}\) level ([Ca\(^{2+}\)_i]) in heart (Harding, Kirschenlohr, Metcalfe, Morris, and Smith, 1989; Frampton, Orchard, and Boyett, 1991) and in smooth muscle (McCarron, McGeown, Reardon, Ikebe, Fay, and Walsh, 1992). Intracellular Ca\(^{2+}\) ions act in a number of specific ways, in-

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*Address correspondence to Dieter J. Pelzer, Membrane Transport and Signaling Group, Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7.
including interaction with Ca\(^{2+}\)-binding proteins such as calmodulin or troponin C, the activation or inhibition of ionic channels (e.g., Hille, 1992), or activation of Ca\(^{2+}\)-dependent enzymes such as protein kinase C (e.g., Nishizuka, 1992) or calmodulin-dependent protein kinase II (e.g., McCarron et al., 1992).

Intracellular Ca\(^{2+}\) also alters the further entry of Ca\(^{2+}\) because of its influence on L-type Ca\(^{2+}\) current (\(I_{\text{Ca}}\)); either stimulation or inhibition of \(I_{\text{Ca}}\) or both have been observed under various conditions which elevate [Ca\(^{2+}\)]\(_i\) (reviewed in McDonald, Pelzer, Trautwein, and Pelzer, 1994). In addition to any effects mediated by an increased [Ca\(^{2+}\)]\(_i\), an increase in stimulation frequency can also alter \(I_{\text{Ca}}\) due to the reduced time spent at diastolic potentials. These two influences act simultaneously during \(\beta\)-adrenergic stimulation of the heart, which elevates intracellular cAMP levels, causing both an increased heart rate and a large enhancement of \(I_{\text{Ca}}\) in the myocardium. The latter occurs even with a fixed stimulation rate (e.g., Hartzell, Méry, Fischmeister, and Szabo, 1991), yet changes in \(I_{\text{Ca}}\) are especially critical at elevated heart rates, because Ca\(^{2+}\) will enter the cell more frequently, further altering steady state [Ca\(^{2+}\)]\(_i\), contractility, and \(I_{\text{Ca}}\) itself.

The force of contraction is well correlated to intracellular Ca\(^{2+}\), and both increase with frequency in mammalian (except for rat) ventricular muscle (cf. Bers, 1991, chapter 8). This relationship draws attention to the modulation of \(I_{\text{Ca}}\), an important source of Ca\(^{2+}\) during rapid stimulation. A direct investigation of the influence of \(I_{\text{Ca}}\) agonists and antagonists on post-rest tension was made by Harrison, Milner, and Boyett (1993) in guinea pig papillary muscle. They identified the negative influence of Ca\(^{2+}\) channel antagonists (Cd\(^{2+}\) and nifedipine) and the positive influence of noradrenaline and the Ca\(^{2+}\) channel agonist Bay K 8644 on the size and rapidity of development of the tension increment occurring with stimulation after a period of rest.

The influences of cAMP on post-rest \(I_{\text{Ca}}\) and tension have also been investigated in guinea pig heart cells by Fedida, Noble, and Spindler (1988b), who noted larger reductions of current in the presence of adrenaline than in controls. They attributed the effect to the larger size of \(I_{\text{Ca}}\), a conclusion also made by Argibay, Fischmeister, and Hartzell (1988) after they observed that large Ca\(^{2+}\) current densities exhibited slower reactivation and were reduced (rather than enhanced) when the stimulus frequency was raised from 0.125 to 2 Hz. Tseng (1988) identified depolarized holding potentials as a detrimental factor in the rate and extent of reactivation of \(I_{\text{Ca}}\), an issue which was explored extensively by Schouten and Morad (1989). The latter authors described the necessity for a negative holding potential (−90 mV) and cAMP in the dialysate, in allowing an enhancement of \(I_{\text{Ca}}\) on switching from 0.1 to 1 Hz in frog ventricular cells. They also showed that after cAMP injection, theophylline, a phosphodiesterase inhibitor, slowed the decay of the \(I_{\text{Ca}}\) enhancement, an effect which was mimicked by injecting cAMP while stimulating at a higher frequency (1 Hz). It was hypothesized that a voltage-dependent inhibition of phosphodiesterase activity allowed cAMP accumulation.

Here we present data which attempt to clarify the relationship between increased frequency and \(\beta\)-adrenergic stimulation, and their interaction in the modulation of \(I_{\text{Ca}}\). We have systematically investigated the frequency-dependent nature of \(I_{\text{Ca}}\) by changing the stimulation rate over a broad range, and here describe some striking
influences of cAMP on the frequency response of $I_{Ca}$. The inverse question is then explored briefly with experiments in which a β-adrenergic agonist was applied while evoking $I_{Ca}$ at high rates. Part of this work has appeared in abstract form (Kasper and Pelzer, 1993).

METHODS

Cell Preparation

Myocytes were dissociated from the ventricles of hearts from adult male guinea pigs (300–500 g), which were killed by cervical dislocation. After aortic cannulation, the coronary circulation was retrogradely perfused at 35°C with a physiological salt (PS) solution (0.5–2 min) containing (in millimolar): 140 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 1.0 MgCl$_2$, 10 Glucose, 10 HEPES, pH 7.4 (NaOH), then with a nominally Ca$^{2+}$-free modified PS solution containing 125 NaCl, 5.4 KCl, 1.0 MgCl$_2$, 10 Glucose, 10 HEPES, pH 7.4 (NaOH), first alone (2–7 min), then with collagenase (30–60 U/ml, 4–12 min), and with a high K$^+$, Ca$^{2+}$-free ‘KB’ incubation solution (2–5 min) containing 80 KCl, 30 KH$_2$PO$_4$, 50 glutamic acid, 20 taurine, 10 Glucose, 10 HEPES, 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), pH 7.4 (KOH). All solutions were continuously bubbled with 100% oxygen. Ventricular tissue was chopped, swirled in four to nine washes of KB solution, and strained (220-μm nylon mesh). Electrical recordings were made for 6 to 18 h after cell isolation. Cell dimensions were measured before patching, using a calibrated graticule in the eyepiece of the inverted microscope (Nikon Diaphot). Cells bathed in PS solution had mean dimensions of 131.0 ± 1.46 (SEM) μm by 19.2 ± 0.35 μm and a mean cell area of 2,515 ± 56.50 μm$^2$, for the 190 cells for which the dimensions were measured.

Water for all uses was distilled, deionized with a milli-Q reverse osmosis system to a resistivity of 18 MΩcm, then pressure filtered (pore size 0.22 μm). Inorganic reagents were from BDH, Calbiochem-Novabiochem Corp. (La Jolla, CA), Aldrich Chemical Company (Milwaukee, WI), and Sigma Chemical Co. (St. Louis, MO). Collagenase was from Yakult (Tokyo, Japan).

Electrical Recordings

The whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used to record currents under voltage clamp conditions (EPC-7 amplifier, List, Darmstadt) and to isolate $I_{Ca}$ through dialysis with Cs$^+$-rich pipette solution. In addition to being rod shaped and appearing relaxed with well-defined striations, the cells which were analyzed did not exhibit transient inward currents on repolarization, a hallmark of Ca$^{2+}$ overload (Fedida, Noble, Rankin, and Spindler, 1987).

Patch pipettes, fabricated from cylindrical thick-walled hard borosilicate glass (Jencons Scientific, Bedfordshire, UK), had resistances of 1.5–3 MΩ when filled with the Cs$^+$-aspartate pipette solution (see below) and exposed to PS solution. The liquid junction potential between the pipette and bath was not corrected. Compensation procedures for pipette capacitance and series resistance ($R_s$) were sometimes employed (70–85% compensation in most cases, generally to a point just below that which caused ringing). Typical values of $R_s$ were 6–11 MΩ. Therefore, uncompensated $R_s$ would not have exceeded 11 MΩ × (1–0.70) = 3.3 MΩ, which causes an error of <5 mV in command voltage for a current of 1,500 pA, which is a typical value for peak Ca$^{2+}$ channel current.

From a holding potential of −80 mV, a 70-ms prepulse to −40 mV was used to inactivate Na$^+$ channel current ($I_{Na}$) before eliciting $I_{Ca}$. Then maximal $I_{Ca}$ was evoked with a 60-ms voltage clamp step to 0 mV, followed by a 40-ms step to −40 mV before returning to −80 mV. In experiments where Ba$^{2+}$ or Na$^+$ carried the current through the Ca$^{2+}$ channels ($I_{Ba}$ or $I_{Ca(NA)}$, respectively), the
holding and test potentials were adjusted in order to compensate the pipette potential for changes in membrane surface potential (Nathan, 1986; Green and Andersen, 1991). The attainment of maximal current near -10 mV was the criterion used for setting the test potential.

Whole-cell currents evoked with continuous voltage-clamp steps were recorded with an FM recorder (Hewlett Packard Co., Palo Alto, CA) onto Ampex tapes. The tape speed was 1.875 or 3.750 in/s, affording a bandwidth passing DC to 0.6 or 1.0 kHz (3 dB down point). Current traces were digitized offline at a sampling rate of 2-5 kHz (usually 3 kHz), and were not leak subtracted. Positive-going capacitance currents are truncated in figures for visual clarity. Currents evoked during very rapid stimulation were all recorded on tape, but only every second or third trace was digitized; synchronicity with real time was preserved in all cases.

Composition of Experimental Solutions

The pH of all bath solutions was adjusted to 7.4 with NaOH, at 20-23°C for room temperature experiments, and at 35°C for solutions used in experiments conducted with a bath temperature of 33-37°C. Cells were bathed in PS solution while patching. Soon after sealing, the bath was switched to a K+-free external solution which had the same composition as PS solution except that CaCl₂ replaced the KCl. When recording Iₙa, the K+-free solution was used with BaCl₂ replacing CaCl₂. For recording Iₜₐₜ(Na), a Ca²⁺-free bathing solution was used which contained (in millimolar): 144 NaCl, 5.4 CsCl, 0.2 or 0.3 MgCl₂, 10 HEPES, 0.33 NaI-I₂PO₄, 10 Glucose, 0.1 EGTA; free [Mg²⁺] = 198 or 297 μM. We found that the addition of this quantity of Mg²⁺ was required to minimize the nonspecific leakage current at the holding potential, which can develop upon switching to the test solution used for recording Iₜₐₜ(Na). In all cases, the pipette solution contained (in millimolar): 110 Cs-Aspartate, 20 CaCl₂, 1 MgCl₂, 4.5 or 5.0 MgATP, 8 HEPES, and pH was adjusted to 7.4 with CsOH. For most of the results presented, 5 mM EGTA was also added; free [Ca²⁺] was estimated as 1-5 pM in early experiments and adjusted to ~10 nM by adding 0.85 mM CaCl₂ in later work, with no difference in results. In some experiments (see Fig. 3 D), only 0.1 mM EGTA was used, and free [Ca²⁺] was adjusted to ~100 nM at pH 7.2 by adding 30 μM CaCl₂ from 15 mM aqueous stock. Where indicated, 50 μM 3-isobuty1-methylxanthine (IBMX) was added as well.

Drugs

Isoprenaline (HCl salt, 1-3 μM, Calbiochem Corp.), a synthetic β-receptor agonist, was added to solutions from 1 mM stock in water. Forskolin (1-3 μM, Calbiochem Corp.) is a direct activator of adenylyl cyclase and was added from 5 or 10 mM stock in DMSO (dimethyl sulfoxide, Sigma Chemical Co.), leaving 0.01-0.06% DMSO (vol/vol) in experimental solutions (i.e., <3% of solution osmolarity came from DMSO). The membrane-permeable cAMP analogue, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP, 150 μM, Boehringer Mannheim Corp., Indianapolis, IN), was added directly to the Cs⁺-substituted PS solution. IBMX (50 μM, Sigma Chemical Co.), a broad-spectrum phosphodiesterase inhibitor, was added to solutions from 5 mM stock in 0.05 % DMSO vol/vol (final DMSO concentration 0.0003% vol/vol). Most solutions were sonicated. Changing the bath solution to a control solution containing DMSO, in the same concentration that was to be used with the drug, caused no artifacts. Bay K 8644 (1.7 μM, Calbiochem Corp.), an agonist of the L-type Ca²⁺ channel, was prepared from a 145 μM stock in DMSO; because this resulted in a fairly high concentration of DMSO (1.2% vol/vol), the same amount of DMSO was always included in the control K⁺-free bath solution. The wash-in time for bath applications of drugs was 30-70 s.

Data Presentation, Analysis, and Statistics

The stimulation rate was always 0.5 Hz as a control rate, and was changed to either 0.1, 0.2, 1, 2, 3, 4, or 5 Hz, then back to 0.5 Hz. The steady state current amplitude of Iₜₐₜ is expressed relative to
0.5 Hz control values, with baseline corrections for rundown where indicated. The means ± SEM of steady state values were plotted against a logarithmic frequency axis. We adopt the terminology that a "positive staircase" is a gradual increase of peak $I_{ca}$ and a "negative staircase" is a gradual decrease. When a positive staircase is preceded by a transient reduction of current below values at the control frequency (0.5 Hz), the transient effect is called depression; a transient increase of current before a negative staircase is called facilitation. The focus of this paper shall be the steady state reduction of Ca$^{2+}$ channel current with rapid stimulation.

Peak Ca$^{2+}$ channel current is the parameter used for all analyses, and in Fig. 6B the integrated Ca$^{2+}$ current is also indicated for validation, because the inactivation rate decreased greatly when isoprenaline was applied after Bay K 8644. A formal comparison was made (not shown) between staircase steady states expressed as normalized peak current or normalized integrated $I_{ca}$; the two analyses were well correlated ($R = 0.7098$), implying validity in the use of $I_{ca}$ amplitude as an index of Ca$^{2+}$ entry in this application. Integrals included the 60-ms test pulse to 0 mV, excluding spikes of capacitance current. Integration was performed using the trapezoid rule at calculation intervals of 0.33 or 0.5 ms (the inverse of the digitization frequencies, 3 and 2 kHz, respectively).

Statistical analysis was carried out using the SAS software package (version 6.08; SAS Institute Inc., Cary, NC) on Dalhousie’s VAX mainframe computer (model 4000-500). Two-way ANOVA was used to compare the effects of frequency under multiple conditions, followed by either one-way ANOVA or else a comparison of all combinations of conditions with Newman-Keuls student t tests at each frequency. One-way ANOVA was used for comparison of the effects of frequency under two experimental conditions, and was followed by t tests at each frequency where pertinent. When comparing multiple pairs of conditions with Newman-Keuls t tests, an absolute criterion of $P < 0.05$ was used for the overall $a$ error. When only two conditions are being compared, the test is identical to multiple t tests. Paired t tests were used where indicated (Fig. 6). For linear regressions, the goodness of fit is indicated by $R$, the square root of the coefficient of determination.

RESULTS

Modulation of Basal Ca$^{2+}$ Channel Current by Stimulation Rate: Effect of Charge Carrier and Temperature

In Fig. 1, the effect of a change in stimulation rate on basal Ca$^{2+}$ channel current is characterized for different charge carriers and temperatures. The decline of Ca$^{2+}$ channel current with high frequency was unaffected by substitution of Ba$^{2+}$ or Na$^{+}$ for Ca$^{2+}$ as charge carrier, but was profoundly enhanced by decreased temperature. Fig. 1A is a comparison of $I_{ca}$, $I_{ba}$, and $I_{ca(Na)}$ current traces obtained at different stimulation rates with the three different charge carriers (Ca$^{2+}$, Ba$^{2+}$, and Na$^{+}$, respectively). Current traces were taken from the steady states of 2, 3, and 4 Hz staircases that were near the mean. In all cases, the data was obtained in the basal state (no drugs) at room temperature (21–25°C, mean = 24.3°C). Each trace has been normalized to the prestaircase peak current at 0.5 Hz. The reduction of $I_{ba}$ amplitude with increased stimulation rate was about the same as $I_{ca}$, and the reduction of $I_{ca(Na)}$, although appearing slightly greater, was not statistically different from the other two conditions (see Fig. 1C below). Fig. 1B shows a representative group of basal $I_{ca}$ traces recorded with a bath temperature of 33–37°C (mean = 35.6°C). Because the inactivation rate in this temperature range is more than double that at room temperature, the time scale used is twice as fast as in Fig. 1A, in order to make the traces distinguishable. The reduction of current with rapid stimulation is much less pronounced than it was at room temperature.
FIGURE 1. Summary of the effects of charge carrier and temperature on the frequency response of Ca$^{2+}$ channel current. (A) Groups of representative current traces at 0.5, 2, 3, and 4 Hz, normalized to the peak value at 0.5 Hz. Ca$^{2+}$ channel current was carried by 1.8 mM Ca$^{2+}$ (left), 1.8 mM Ba$^{2+}$ (middle), or ~145 mM Na$^+$ (right). The traces of $I_{Ca}$ are all from one cell; the others are from two cells each. All records were obtained at room temperature. (B) Group of basal $I_{Ca}$ traces obtained at 36°C. Note the difference in time scale; clamp pulses lasted 60 ms in all cases. (C) Frequency response curves for Ca$^{2+}$ channel current carried by Ca$^{2+}$ (filled circles), Ba$^{2+}$ (hollow circles), or Na$^+$ (hollow triangles); all data recorded at room temperature. Points represent means (±SEM) of steady state Ca$^{2+}$ channel currents normalized to values at 0.5 Hz before each frequency change. The respective numbers of frequency changes at 0.1, 0.2, 1, 2, 3, 4, and 5 Hz were as follows: 5, 3, 32, 30, 23, 14, and 8 for basal $I_{Ca}$; 5, 3, 4, 4, 3, and 4 for basal $I_{Ba}$; and 3, 5, 8, 5, 6, and 4 for basal $I_{Ca(Na)}$. The
Fig. 1 C shows the mean ± SEM steady state values of peak basal $I_{ca}$, $I_{ba}$, and $I_{Ca\text{[Na]}}$, normalized to their respective values at the control frequency (0.5 Hz) and plotted against a logarithmic axis of the test frequency. Graphs like this one shall be referred to as frequency response curves. At room temperature, peak Ca$^{2+}$ channel current begins to fall off appreciably at 1–2 Hz, and at 5 Hz has fallen to <25% of control. The inset to Fig. 1 C shows the arithmetic differences between the mean steady states of $I_{ca}$ and $I_{ba}$ and between $I_{ca}$ and $I_{Ca\text{[Na]}}$, at each frequency. The maximal differences of normalized current amounted to only about a 10% greater reduction of $I_{Ca\text{[Na]}}$ than $I_{ca}$ at 2 Hz, and a 10% lesser reduction of $I_{ba}$ than $I_{ca}$ at 0.1 and 5 Hz.

Fig. 1 D compares the frequency response of basal $I_{ca}$ in the two temperature ranges (21–25°C and 33–37°C). The decline of $I_{ca}$ at room temperature exceeds the decline of $I_{ca}$ in the 33–37°C range, at frequencies >1 Hz. The arithmetic difference between the two curves is plotted in the inset; the magnitude of the difference rises monotonically with stimulation rate in the 2–5 Hz range, inclusive. The difference was fairly large; for instance, the mean steady state reduction of $I_{ca}$ was 30 percentage points larger with a switch from 0.5 to 5 Hz at room temperature, respective to the mean inhibition recorded with a bath temperature of 33–37°C.

**Modulation by Stimulation Rate of cAMP-elevated $I_{ca}$**

The reduction of peak $I_{ca}$ with increasing stimulation rate is enhanced in the presence of maximal concentrations of drugs which elevate cAMP. This is demonstrated in the $I_{ca}$ time courses shown in Fig. 2. Changes in stimulation rate were imposed when the cell was in the basal state and after isoprenaline, forskolin, or CPT-cAMP had been applied at concentrations which produced maximal increases of $I_{ca}$ at 0.5 Hz. Fig. 2 A shows that the frequency-induced reduction of peak current was much greater after 1 μM isoprenaline had elevated the current, as compared to the smaller basal staircases in the same cell. There is a greater reduction in absolute current (picoamperes), as well as a greater fractional decrease relative to 0.5 Hz controls. We have called this effect "extra inhibition" to differentiate it from the lesser amount of reduction which is seen when rapidly evoking basal current. The records in Fig. 2, B and C, demonstrate extra inhibition measured in the pres-

inset is a plot of the arithmetic differences between frequency response curves (between $I_{ca}$ and $I_{ba}$, circles; between $I_{ca}$ and $I_{Ca\text{[Na]}}$, triangles). The error bars were calculated using the identity that the variance of a sum or difference is the sum of the variances of the addends or minuend and subtrahend (Montgomery, 1991: 72). None of the frequency response curves differed from each other, as judged by two-way ANOVA ($P = 0.6359$). (D) Frequency response curves for basal $I_{ca}$ at room temperature (21–25°C, filled circles) and in the 33–37°C temperature range (hollow triangles). The room temperature curve is repeated from C. The two curves were statistically different ($P = 0.0012$, one-way ANOVA; $P$ values from t tests, at 2, 3, 4, and 5 Hz, are 0.038, 0.012, 0.040, and 0.0003, respectively). The respective numbers of frequency changes at 0.1, 0.2, 1, 2, 3, 4, and 5 Hz were: 5, 3, 30, 30, 28, 14, and 8 for basal $I_{ca}$ at 21–25°C; and 5, 5, 5, 6, 6, 5, and 6 for basal $I_{ca}$ at 33–37°C. The inset is a plot of the arithmetic differences between the two curves, with error bars calculated as for the inset in C.
FIGURE 2. Time courses of peak $I_{ca}$ at room temperature, demonstrating that negative staircases of $I_{ca}$ are greatly enhanced after high concentrations of isoprenaline (Iso, 1 μM), forskolin (Fsk, 3 μM), or CPT-cAMP (150 μM) were applied. Stimulation rate is 0.5 Hz except where labeled otherwise. (A) Negative staircases of $I_{ca}$ in the basal state and after Iso caused a 173% enhancement of current at 0.5 Hz. Cell sr152, 22.5°C. (B) Time course of peak $I_{ca}$ in the basal state and after an 87% increase with Fsk applied at 0.5 Hz, recorded from cell sr133 at 22.5°C. In the first basal staircase there was a transient facilitation of 6.8% on switching from 0.5 Hz to 2 Hz, followed by a negative staircase; switching back to 0.5 Hz caused transient depression (0.926 fold of 2 Hz current). (C) Time course of peak $I_{ca}$ from cell sr161 at 24.5°C. Staircases of basal current were followed by the application of 150 μM CPT-cAMP, which elevated the current by 68%. After this maximal rise there was steady run-
ence of 3 μM forskolin or 150 μM CPT-cAMP, respectively. Note that in the presence of CPT-cAMP, the extra inhibition of $I_{ca}$ was much less pronounced than it was with forskolin or isoprenaline, as seen both visually (Fig. 2 C) and on average (see Fig. 3).

With Fig. 3, we compare the frequency response of basal and up-modulated $I_{ca}$, both at room temperature and in the 33–37°C range. In either temperature range, the frequency-induced depression of $I_{ca}$ was more pronounced after elevation of cAMP. In Fig. 3 A, groups of current traces are shown from the steady states of representative 2-, 3-, and 4-Hz staircases at room temperature, all normalized to the control current at 0.5 Hz just before the frequency change. Normalized basal current is sequentially less with an increase in stimulation rate to 2, 3, or 4 Hz, an effect which was enhanced by cAMP-elevating drugs, the strongest effect being produced by forskolin > isoprenaline > CPT-cAMP.

Fig. 3 B shows the frequency response curves obtained under basal conditions, or with isoprenaline, forskolin, or CPT-cAMP. At 1–4 Hz inclusive, $I_{ca}$ exhibits an extra component of reduction when cells were exposed to these drugs which elevate cAMP. The magnitude of this extra inhibition can be seen in the graph of the arithmetic differences (Fig. 3 B, inset) between the frequency response curves of basal $I_{ca}$ and $I_{ca}$ with each drug. At 5 Hz, the steady states were very similar for $I_{ca}$ under all conditions shown, probably because the current was very small at 5 Hz and because negative values were arbitrarily excluded.

Fig. 3 C shows some normalized steady state traces of $I_{ca}$ recorded in the 33–37°C range, for basal $I_{ca}$ and $I_{ca}$ up-modulated by 1 μM isoprenaline. For visual clarity, only traces at 0.5 and 3 Hz are shown. Again, with 1 μM isoprenaline there is a large extra component of reduction induced by an increase in stimulation rate from 0.5 to 3 Hz.

Fig. 3 D shows the frequency response curves for $I_{ca}$ at 33–37°C, both in the basal state and with 1–3 μM isoprenaline. There was not a floor effect at 5 Hz (cf. Fig. 3 B), because the steady states of basal $I_{ca}$ were not close to zero in this temperature range. The extra inhibition of current with isoprenaline was evident at higher frequencies (2–5 Hz) at 33–37°C than at room temperature (1–4 Hz at 21–25°C) and down (3%/min), which has been baseline corrected in that part of the time course for visual comparability. Steady state values of staircases shown in A–C were as follows:

| Panel | Frequency | Duration | SS  | Drug | Duration | SS |
|-------|-----------|----------|-----|------|----------|----|
| A     | 1 Hz      | 75 s     | 1.00| Iso  | 78 s     | 0.746|
|       | 2 Hz      | 76 s     | 0.740|      | 75 s     | 0.488|
| B     | 2 Hz      | 68 s     | 0.720| Fsk  | 72 s     | 0.391|
|       | 4 Hz      | 66 s     | 0.247|      | 70 s     | 0.075|
| C     | 1 Hz      | 93 s     | 1.00| CPT-cAMP | 75 s | 0.959|
|       | 2 Hz      | 74 s     | 0.708|      | 71 s     | 0.731|

SS refers to the steady state amplitude of current expressed as a fraction of the pre-staircase amplitude at 0.5 Hz.
FIGURE 3. Summary of the effects of cAMP elevation on the frequency response of $I_{Ca}$. Data shown in A and B were obtained at 21-25°C, and C and D show comparable data obtained at 33-37°C. (A) Normalized representative $I_{Ca}$ traces at 0.5, 2, 3, and 4 Hz in the steady state for basal $I_{Ca}$, and after enhancement (at 0.5 Hz) by isoprenaline (Iso), forskolin (Fsk), or CPT-cAMP. Traces were taken from two cells for each group of traces. (B) Frequency response curves for $I_{Ca}$ in the basal state (filled circles) and after its enhancement (at 0.5 Hz) to 3.13 ± 0.28 (21 cells), 2.59 ± 0.21 (16), or 2.39 ± 0.29 (12) fold of basal current by 1-3 µM Iso (hollow circles), 1-3 µM Fsk (hollow squares), or 150 µM CPT-cAMP (hollow triangles), respectively. Analysis of variance (ANOVA, two-way) confirmed an effect of these drugs on the frequency response of $I_{Ca}$ ($P = 0.0010$); the results of one-way ANOVA tests for each condition (also for D) were as follows:

| Panel | Drug     | $P$ (ANOVA) | Significant tests ($P < 0.05$) |
|-------|----------|-------------|--------------------------------|
| B (21-25°C) | Iso      | <0.0001     | 1, 2, 3, 4, Hz                  |
|       | Fsk      | <0.0001     | 1, 2, 3, 4 Hz                   |
|       | CPT-cAMP | 0.0244      | 4 Hz only                       |
| D (33-37°C) | Iso      | 0.0065      | 3, 4, 5, Hz ($P = 0.0537$ at 2 Hz) |
was of a greater magnitude (Fig. 3 D, inset) in the higher temperature range. Shown superimposed on the frequency response curves are the mean ± SEM values of staircase steady states recorded with only 0.1 mM EGTA (rather than 5 mM) in the dialysate; the similarity is evident, and indicates that within this concentration range, the presence of EGTA does not influence the Ica staircase. However, in most preparations, the 5 mM EGTA prolonged the stable recording time in each cell.

**Sodium Loading with Repetitive Stimulation: Lack of Effect on Frequency-induced Extra Inhibition**

Although the cells used here were dialyzed with Na+-free pipette solution, the Na+ channel current elicited with the prepulse would cause intracellular Na+ accumulation during stimulation (e.g., Boyett, Hart, and Levi, 1987). Lee and Levi (1990) used the Na+ sensitive fluorescent dye, SBFI, to measure intracellular sodium activity in rabbit and rat heart cells, and found an elevation of ~100% with rapid stimulation (2 Hz, 350-ms clamp steps from −90 to 0 mV). Elevated intracellular Na+ reduces the gradient for Ca2+ extrusion via Na+-Ca2+ exchange, and subsequently enhances the background Ca2+ level.

The results of a series of experiments obtained from a depolarized holding potential (−40 mV, no prepulse; not shown) were compared to those obtained with a prepulse that elicited Na+ entry (Fig. 3). Besides avoiding the fast Na+ current, the holding potential of −40 mV may also cause a slow voltage-dependent reduction of current (Schouten and Morad, 1989), and reduces the efficacy of Na+-Ca2+ exchange (Kimura, Miyamae, and Noma, 1987) and reactivation in heart cells (Argibay et al., 1988, in frog; Tseng, 1988, in dog and guinea pig; Peineau et al., 1992, in guinea pig; Richard et al., 1993, in rat). Regardless, the extra inhibition with 1 μM isoprenaline was still observed with a holding potential of −40 mV (n = 36 frequency changes in total).
FIGURE 4. Summary of the response of $I_{\text{Na}}$ to changes in stimulation rate, both in the basal state and with isoprenaline (Iso, 1–3 μM) present. Stimulation rate is 0.5 Hz except where labeled otherwise. (A) Time course of $I_{\text{Na}}$ from cell s177, recorded at 22°C. Stimulation at 3 Hz lasted 90 s for basal $I_{\text{Na}}$, and the steady state of the 3-Hz staircase is 0.435-fold of $I_{\text{Na}}$ at 0.5 Hz. After 3 μM Iso had enhanced the current at 0.5 Hz to 2.81-fold of control (mean ± SEM was 2.00 ± 0.27-fold in seven cells), an 89-s period of stimulation at 3 Hz was imposed, causing a staircase to 0.788-fold of control current. (B) Normalized representative current traces from the steady states of staircases that were near the mean values, for $I_{\text{Na}}$ in the basal state and with 3 μM Iso. Traces were taken from two cells in all. (C) Frequency response curves for $I_{\text{Na}}$, in the basal state (solid circles) and after up-modulation to 2.00 ± 0.27-fold (mean ± SEM in seven cells stimulated at 0.5 Hz) by 1–3 μM Iso (solid triangles). The curves were indistinguishable statistically ($P = 0.9511$, one-way ANOVA). The inset shows the arithmetic difference between the curves; for error bar calculation, see Fig. 1 C. All of the data were obtained at room temperature; the basal curve was also shown in Fig. 1 C. The respective numbers of frequency changes at 0.1, 0.2, 1, 2, 3, 4, and 5 Hz were as follows: 3, 5, 8, 5, 4, 6, and 4 for basal $I_{\text{Na}}$; and 3, 4, 5, 4, 5, 3, and 4 with Iso.
Modulation by Stimulation Rate of cAMP-elevated Na+ Current through Ca2+ Channels

Isoprenaline did not enhance the frequency-induced reduction of Ca2+ channel current when Na+ rather than Ca2+ was used as the charge carrier. Fig. 4 summarizes experiments carried out with the same protocol as used in Fig. 3, except that Na+ was used as the charge carrier. The time course of peak ICa(Na) shown in Fig. 4A, demonstrates that a period of stimulation at 3 Hz causes similar reductions of basal and isoprenaline-stimulated ICa(Na).

Fig. 4B shows normalized current traces at 0.5, 2, 3, and 4 Hz from staircases that had steady states near the mean for each frequency. The relatively large holding current observed under this recording condition is a general finding (e.g., Matsuda, 1986). The reduction of ICa(Na) induced by high frequency stimulation is strikingly similar in either the basal state or in the presence of 3 μM isoprenaline.

Table 1

| Frequency (Hz) | Basal Steady state ICa | Isoprenaline Steady state ICa | P value |
|---------------|------------------------|------------------------------|--------|
| 1.0           | 0.9260 ± 0.0121 (5)    | 0.9566 ± 0.0596 (3)          | 0.6996 |
| 2.0           | 0.7512 ± 0.0286 (5)    | 0.8156 (1)                   | —      |
| 3.0           | 0.5416 ± 0.0569 (6)    | 0.5204 ± 0.0659 (3)          | 0.7879 |
| 4.0           | 0.4058 ± 0.0648 (3)    | 0.4667 ± 0.0471 (2)          | 0.6846 |

Steady state values represent mean ± SEM (n) of ICa normalized to 0.5-Hz controls. The P values are from Newman-Keuls's t tests; one-way ANOVA on the entire pool of available data also indicated that there was not a statistically significant difference (P = 0.8603) between the basal condition and 1–3 μM isoprenaline. All data recorded with a bath temperature of 21–25°C.

Fig. 4C shows the frequency response curves at room temperature for ICa(Na) in the basal state and with 1–3 μM isoprenaline, and the inset is a plot of the arithmetic differences between the two curves. Despite the doubling, on average, of ICa(Na) at 0.5 Hz by isoprenaline, the attenuation of current by frequency was nearly identical to that under basal conditions. There was no extra inhibition of ICa(Na), as the frequency response curves were visually nearly identical. Confirmatory results were obtained for ICa(Na) at 33–37°C (not shown; P = 0.7861, total n = 14 for basal current and 8 with 1–3 μM isoprenaline), and with Ba2+ as charge carrier at room temperature (summarized in Table 1).

Modulation by Stimulation Rate of cAMP-elevated ICa: Effect of Phosphodiesterase Inhibition with IBMX

When ICa was enhanced by CPT-cAMP, the frequency response curve, although still showing more attenuation than the basal curve, was much closer to the basal curve.
FIGURE 5. Effects of extracellular IBMX on the frequency response of $I_{ca}$. Stimulation rate is 0.5 Hz except where labeled otherwise. (A) Time course of a 166% increase in peak $I_{ca}$ at 0.5 Hz, with the application of 50 μM IBMX to the bath. Also shown is a 150-s period of 3-Hz stimulation. Notice the lag period before the beginning of the negative staircase. Cell sr302, 36°C. (B) Normalized current traces recorded at 36.5°C, taken from 3-Hz staircases whose steady states were near the mean, for the basal condition and with IBMX. The arrows beside the current traces mark the peak values. (C) Frequency response curves for $I_{ca}$ in the basal state (solid circles) and $I_{ca}$ enhanced to 2.34 ± 0.22-fold of $I_{ca}$ at 0.5 Hz (mean ± SEM in 22 cells stimulated at 0.5 Hz) by 50 μM extracellular IBMX (solid triangles). The inset is a plot of the arithmetic difference between the two curves; for error bar calculation, see Fig. 1 C. The respective numbers of frequency changes at 0.1, 0.2, 1, 2, 3, 4, and 5 Hz (at 36°C) were as follows: 5, 5, 6, 5, 6, 5, 7, 7, and 6 with extracellular IBMX. A comparison of the frequency response curves for basal $I_{ca}$, $I_{ca}$ with intracellular IBMX, and $I_{ca}$ with extracellular IBMX was made with two-way ANOVA, and no statistical differences were found in the frequency response curves of $I_{ca}$ ($P = 0.4144$).
than were the forskolin or isoprenaline curves. Thio analogues of cAMP are hydrolyzed more slowly than the endogenous form whose production is induced by isoprenaline or forskolin, and in a sense these analogues are competitive inhibitors of phosphodiesterase; CPT-cAMP competes with $[^3H]cAMP$ for hydrolysis by phosphodiesterases with $\sim 1/30$ the affinity of cAMP (Beebe, Beasley-Leach, and Corbin, 1988).

To investigate the possible involvement of phosphodiesterases, 50 μM IBMX was added to the bath. Intracellular IBMX did not elevate $I_{Ca}$ (cf. Simmons and Hartzell, 1988), possibly because this hydrophobic drug may leave the cell faster than it can be supplied by dialysis, or the phosphodiesterase which was inhibited by extracellular IBMX might be harder to access with IBMX applied intracellularly (Fischmeister and Hartzell, 1987). Hence, both intracellular and extracellular applications were investigated. All of the data with IBMX was obtained with a bath temperature of 33–37°C, and was compared to basal data obtained in the same temperature range. While evoking $I_{Ca}$ at 0.5 Hz, bath applications of 50 μM IBMX caused a similar increase in $I_{Ca}$ as isoprenaline (1–3 μM isoprenaline at 33–37°C: 2.65 ± 0.25-fold [12 cells]; 50 μM IBMX at 33–37°C: 2.34 ± 0.22-fold [22 cells]).

Fig. 5 shows that the extra inhibition of $I_{Ca}$ was not induced by high frequency when cAMP was elevated with IBMX. In Fig. 5 A, the 130-s period of stimulation at 3 Hz reduced $I_{Ca}$ by only 10.5%. In Fig. 5 B, the normalized current traces show the very similar steady state values of $I_{Ca}$ at 3 Hz, and Fig. 5 C shows the similar frequency response curves of $I_{Ca}$ in the basal state and with 50 μM extracellular IBMX. The basal and IBMX curves are essentially superimposable, except for a slight extra reduction of $I_{Ca}$ at 5 Hz with IBMX, where the value was intermediate between the basal and isoprenaline values.

**Effect of Recording Time: Reproducibility**

Despite the advantages of controlling the intracellular ionic milieu and gaining good control of voltage with the large-bore access to the cell, dialysis with a patch clamp pipette may affect the results in some situations. The influence of time spent recording (i.e., dialysis time) was assessed by analyzing the reproducibility of staircase steady states at the same frequency in a given cell under the same condition. Repetitions were separated by 3–29 min in time, and in 47 such pairs of staircases the percentage difference between steady states had an average absolute value of $6.07 \pm 0.85\%$ (SEM); a Gaussian fit to the raw distribution of percent differences between repetitions had a mean ± SD of 0.16 ± 4.14%. The 47 paired comparisons consisted of 40 staircases that were second repetitions of the same test frequency in the same cell, and seven that were third repetitions within the same cell; each repeated measure was compared to the first staircase in the series. The good reproducibility indicates that the recording time did not generally alter the frequency response data, and did not cause the steady states to change due to recording time rather than to applied drugs. The zero mean of the Gaussian distribution indicates that there was not a trend for either a progressive increase or a progressive decrease of staircase steady states with repetitions later in time.
**Extra Inhibition Is Not Caused by the Increased Size of \( I_{\text{Ca}} \) Alone: Requirement for cAMP Elevation**

The results presented above indicate that rapid stimulation reduces cAMP-elevated \( I_{\text{Ca}} \) moreso than basal \( I_{\text{Ca}} \), except when phosphodiesterases are inhibited with IBMX. However, the applied drugs had elevated cAMP and \( I_{\text{Ca}} \) simultaneously, and we next attempted to separate the influences of these two factors. The approach taken was to measure steady states of \( I_{\text{Ca}} \) staircases at 3 Hz, under three sequential conditions: (a) basal; (b) with the \( Ca^{2+} \) channel agonist Bay K 8644 (1.7 \( \mu \)M); and (c) with isoprenaline (3 \( \mu \)M), applied as a mixture with Bay K 8644, which would elevate cAMP levels but at this point provided only a small further increment in peak current. Test frequencies other than 3 Hz were not investigated in this series of experiments. Fig. 6 shows that an elevation of cAMP levels, not only an elevation of \( I_{\text{Ca}} \), is required to induce the extra component of \( I_{\text{Ca}} \) reduction by high-frequency stimulation.

Fig. 6 A shows representative raw current traces from the steady states of \( I_{\text{Ca}} \) at 0.5 and 3 Hz, all recorded from one cell at 37\(^\circ\)C. It is evident from the current traces that the \( I_{\text{Ca}} \) staircase was small for the basal and Bay K 8644 conditions, but quite large after the addition of isoprenaline.

While stimulating at 0.5 Hz, Bay K 8644 elevated \( I_{\text{Ca}} \) to 2.23 ± 0.29 (mean ± SEM, \( n = 5 \)) fold of basal current amplitude. Isoprenaline (solution c above) caused a further elevation of 12.8 ± 5.5% (mean ± SEM, \( n = 5 \)) in peak \( I_{\text{Ca}} \). Despite the small change in peak current with isoprenaline applied after Bay K 8644, the inactivation rate was slowed considerably, so that \( Ca^{2+} \) influx was actually increased much more than 12.8%. To account for this additional \( Ca^{2+} \) influx the integrals were calculated for the current traces in Fig. 6 A, and are plotted in Fig. 6 B. These calculations confirmed that the actual \( Ca^{2+} \) entry during the 60-ms test pulse was indeed much larger with both isoprenaline and Bay K 8644 in combination, than with Bay K 8644 alone. As expected, integrated \( I_{\text{Ca}} \) at 3 Hz rises as a function of that at 0.5 Hz, except that the value at 3 Hz is less than that at 0.5 Hz (i.e., it lies below the line of equality). This was the case for basal \( I_{\text{Ca}} \) and in the presence of Bay K 8644. However, after isoprenaline caused a large further increase of the integral at 0.5 Hz, the steady state integral at 3 Hz was only about as large as that measured with Bay K 8644 alone. Hence, the 3-Hz integral with isoprenaline present had a much lower value than would have been predicted by extrapolating the first two points. Note that the \( Ca^{2+} \) entry was still much greater than for basal current in either case. Analyzing the integral of \( I_{\text{Ca}} \) rather than peak current yielded results that were consistent with the presentation of extra inhibition of peak \( I_{\text{Ca}} \) with high-frequency staircases in the presence of drugs which elevate cAMP.

Fig. 6 C is a plot of the steady states of 3-Hz staircases of basal \( I_{\text{Ca}} \), then after exposure of the cell to Bay K 8644 first alone then in combination with isoprenaline. In the presence of Bay K 8644, which elevates \( I_{\text{Ca}} \) without elevating cAMP levels, the \( I_{\text{Ca}} \) staircase at 3 Hz was comparable to that of basal current from cells bathed in the vehicle alone (1.2% DMSO). After the application of a maximal concentration of isoprenaline in combination with Bay K 8644, \( I_{\text{Ca}} \) staircases at 3 Hz were significantly larger than those in the basal state (with 1.2% DMSO) or with Bay K 8644 alone,
Figure 6. Effects of Bay K 8644 (BayK, 1.7 μM), both alone and in combination with isoprenaline (Iso, 3 μM), on the 3-Hz staircase of $I_{Ca}$. (A) Current traces from cell sr40B7, recorded at 37°C; steady state traces at 0.5 and 3 Hz are indicated for basal $I_{Ca}$ and $I_{Ca}$ after exposure of the cell to BayK then a mixture of BayK and Iso. (B) Integrals of the current traces shown in A. The filled circle is for basal $I_{Ca}$, the filled square is for BayK, and the filled triangle is for the mixture of BayK and Iso. (C) The steady states of peak $I_{Ca}$ staircases at 3 Hz, expressed relative to 0.5-Hz controls in each case. The hollow circles show the results of five individual runs of the full protocol, the filled circles are mean ± SEM values (n = 5 each), and the filled triangles are comparison values taken from Fig. 3 D for 3-Hz steady states of $I_{Ca}$ in the basal state and with 1–3 μM Iso. The mean with Bay K 8644 was no different than with the vehicle alone (1.2% DMSO; $P = 0.3739$, paired $t$ test). With the mixture of Iso and Bay K 8644, the mean was very similar to that with Iso alone, and was significantly different from the mean with DMSO alone ($P = 0.0027$, paired $t$ test) or the mean with Bay K 8644 alone ($P = 0.0098$, paired $t$ test). The temperature was 33–37°C in all cases.
and were very similar to those with isoprenaline alone. The effect is most likely due to elevated cAMP levels arising from isoprenaline application.

Isoprenaline Applied at High Frequencies

To study explicit and specific modulation by drugs while excluding the effects of channel inactivation (cf. Fedida, Noble, and Spindler, 1988a), or cumulative mechanical effects in tissue preparations (cf. Koch-Weser and Blinks, 1963), much car-

![Figure 7](image)

**Figure 7.** Enhancement of $I_{Ca}$ by 1 μM isoprenaline (Iso) during rapid stimulation. Stimulation rate is 0.5 Hz except where labeled otherwise. The protocol for both experiments was to stimulate the cell at 0.5 Hz for the first 9 min after achieving the whole-cell configuration; then the stimulation rate was increased to the test frequency. 3 min later, 1 μM Iso was applied to the bath. (A) Test frequency was 3 Hz; there was an initial transient facilitation of 36.1% followed by a negative staircase of basal $I_{Ca}$. Cell st094, 24°C. (B) Test frequency was 1 Hz; transient facilitation of 11.3% developed initially at 1 Hz. Cell st098, 23°C.

diac electrophysiology has been investigated while stimulating at extremely low (0.033–0.1 Hz) to moderate (0.5–1 Hz) rates. Hence, it is important to show that at the stimulation rates used here, there can still be enzymatic modulation of $I_{Ca}$ in our preparations. To demonstrate this, 1 μM isoprenaline was applied to cells while evoking $I_{Ca}$ at high frequencies (1–3 Hz); examples of these experiments are shown in Fig. 7.
In Fig. 7 A, the application of isoprenaline caused a 410% increase in 3-Hz current. A disproportionately large recovery or enhancement of current was attained after relieving the rapid stimulation; the 0.5-Hz current was ~300% larger than the control current at that rate, after the frequency was changed back in the continued presence of isoprenaline. In Fig. 7 B, there was a 146% increment in 1-Hz current with isoprenaline application; the final current at 0.5 Hz (with isoprenaline) was 123% greater than basal \( I_{Ca} \) at 0.5 Hz, with no correction for rundown. The actual percent rise depended on the size of the basal staircases.

In contrast to the clear enhancement of \( I_{Ca} \) with isoprenaline, obtained even while stimulating the cell at 1–3 Hz (Fig. 7), an examination of Fig. 2 B (cell sr133, \( I_{Ca} \) staircases with forskolin) reveals that the 4-Hz current with forskolin reached a steady state value which fell below the 4 Hz steady state for basal current (absolute current, in picoamperes). This seems counterintuitive in light of the results shown in Fig. 7, because one might expect that if forskolin or isoprenaline was applied (perhaps even at 4 Hz), \( I_{Ca} \) would go up, not down. This effect is assessed in Fig. 8, which is a plot of the ratio of: (a) amplitude of \( I_{Ca} \) in the steady state of a staircase in the presence of isoprenaline, forskolin, or CPT-cAMP, and (b) amplitude of basal \( I_{Ca} \) in the steady state of a staircase at the same test frequency.

It is evident from Fig. 8 that most up-modulated currents do not fall below their corresponding basal values measured at the same frequency (these would have a value <1.0 on the ordinate axis). Such a situation arose primarily at 2, 3, or 4 Hz, and in a minority of the distribution. Hence, at most frequencies, the elevation of cAMP causes a net enhancement of \( I_{Ca} \), even though rapid pulsing causes a powerful inhibition of \( I_{Ca} \) due to phosphodiesterase activation.

**DISCUSSION**

*Strong Negative Influence of High Frequency on \( I_{Ca} \)*

Here we have shown a consistent negative frequency staircase of L-type \( Ca^{2+} \) current in guinea pig ventricular heart cells. Transient facilitation (see Methods for terminology, and Figs. 2 B and 7 for examples) occurred in some cases, but the incidence and strength of this effect was variable (for review, see McDonald et al., 1994). We have recently shown that facilitation could be modulated by the same factors which affect negative staircases (temperature, frequency), but also by manipulations of holding potential or dialysate \( Ca^{2+} \), elevation of cAMP by moderate concentrations of isoprenaline, and phosphodiesterase inhibition with IBMX. Facilitation and its incidence are comprehensively considered elsewhere (Kaspar and Pelzer, 1995), and its origin and functions are still being elucidated by several groups.

*Temperature Dependence of Basal \( I_{Ca} \) Staircases*

The temperature sensitivity of reactivation may generally explain the large effect of temperature on the basal \( I_{Ca} \) frequency response curve (Fig. 1 D). The reactivation of \( I_{Ca} \) in mammalian heart cells is faster at negative potentials (~80 to ~100 mV as compared to ~40 to ~50 mV), and is at least fourfold slower at room temperature than at 37°C (see Pelzer, Pelzer, and McDonald, 1990, and references therein). Re-
FIGURE 8. Net elevation of $I_{cA}$ by drugs that elevate cAMP, at various frequencies. (A) Calculated ratios of up-modulated to basal $I_{cA}$ for room temperature staircases in the presence of 1–8 µM isoprenaline, 1–3 µM forskolin, or 150 µM CPT-cAMP. Drugs were applied while evoking $I_{cA}$ at 0.5 Hz in all cases. At a given frequency, the amplitude $I_{cA}$ in the steady state is called $I_{up}$ when the frequency change was made in the presence of drug, and $I_{bas}$ for the basal condition. The ratio of currents ($I_{up} / I_{bas}$) indicates the relative current that would be expected if the drug was applied while stimulating at the staircase frequency rather than at 0.5 Hz (cf. Fig. 7). To minimize the influence of rundown, this ratio of currents ($I_{up} / I_{bas}$) was estimated from the ratio of normalized steady state values ($SS_{up} / SS_{bas}$): Ratio = $I_{up} / I_{bas}$ = ($SS_{up} / SS_{bas}$) × (Response), where $SS$ is the ratio of $I_{cA}$ at the test frequency to $I_{cA}$ at 0.5 Hz, in the presence of drug for $SS_{up}$, and in the basal state for $SS_{bas}$. "Response" refers to the ratio of up-modulated to basal current at 0.5 Hz at the time of the frequency change (not the maximal response observed in the cell). Hence, for example, at 5 Hz the equation above reduces to:
activation mechanisms are not critical at frequencies <1 Hz, because recovery of $I_{Ca}$ from inactivation is complete in various species within a few hundred milliseconds (e.g., Argibay, Fischmeister, and Hartzell, 1988; Tseng, 1988; Peineau, Garnier, and Argibay, 1992; Richard, Charnet, and Nerbonne, 1993). The observation of a similar effect of increased temperature on staircases as on reactivation (i.e., less reduction or faster recovery from previous stimuli) suggests that reactivation is an important factor in the frequency response of basal $I_{Ca}$. The temperature sensitivities of overlapping pump or exchange currents and enzymes might also contribute.

**Frequency Response of Basal $I_{Ba}$ and $I_{Ca(Na)}$ in Comparison to Basal $I_{Ca}$**

Various established mechanisms could be involved in modifying whole-cell current, such as channel recruitment (Tsien, Bean, Hess, Lansman, Nilius, and Nowycky, 1986) and an ultra-slow type of voltage-dependent inactivation. The latter takes place over several seconds, occurs even when Ba$^{2+}$ is the charge carrier, and has been suggested to underlie the frequency staircase of basal $I_{Ca}$ (Zang, Honjo, Kirby, and Boyett, 1991). Zang et al. (1991) reported that the slowest time constant (5.4 s) of a negative $I_{Ca}$ staircase was similar to the slowest time constant of $I_{Ba}$ inactivation during a 20-s voltage clamp pulse. They suggested that an ultraslow, voltage-dependent inactivation accumulates when the cell’s voltage is kept at positive potentials for a large percentage of time, as would occur with an increase in stimulation rate. Hence, the effects of positive voltage may largely account for the basal staircase of Ca$^{2+}$ channel current, regardless of Ca$^{2+}$ entry.

The reduction of basal Ca$^{2+}$ channel current with an increase in stimulation frequency was the same (both visually and statistically, see Fig. 1) when either Ca$^{2+}$, Ba$^{2+}$, or Na$^{+}$ was used as the charge carrier. Hence the response to stimulation rate was influenced neither by the different mechanisms of permeation through the Ca$^{2+}$ channels nor by the size of the current relative to $I_{Ca}$ [$I_{Ba}/I_{Ca} = 6.69 \pm 0.67$ (13 cells) and $I_{Ca(Na)}/I_{Ca} = 2.87 \pm 0.67$ (8), all at 0.5 Hz].

The equivalent frequency-induced inhibition with Ca$^{2+}$, Ba$^{2+}$, or Na$^{+}$ as charge carrier seemed contradictory to the effects of Ca$^{2+}$-dependent inactivation, but its influence might have been lessened by the use of short clamp pulses. Alternatively, there could be an offsetting effect of altering the membrane’s surface charge when recording $I_{Ca(Na)}$. In models of surface potentials, ions can either bind to or screen...
surface charges (e.g., Nathan, 1986; Green and Andersen, 1991). There is likely a very small effect of charge screening however, because the divalents (Ba$^{2+}$ and Ca$^{2+}$) screen equally well, and bind surface charges more strongly (Ca$^{2+}$ $\gg$ Ba$^{2+}$) than ions contained in the bath solution used for recording $I_{\text{Ca(Na)}}$ (see Nathan, 1986; Kass and Krafte, 1987). Because the curves did not differ statistically, the small deviations between curves most likely reflect experimental scatter rather than genuine differences.

Assessment of Frequency Response with Bay K 8644

Because of the antagonistic effects of Bay K 8644 on $I_{\text{Ca}}$ at mildly positive voltages, it has been suggested that there should be a large negative effect of high frequency stimulation in the presence of the drug, as the voltage is depolarized for a large percentage of time at high frequencies (e.g., Sanguinetti, Krafte, and Kass, 1986; Hadley and Hume, 1988). Because Fig. 6 demonstrates that Bay K 8644 does not enhance the frequency-induced reduction of $I_{\text{Ca}}$ under our conditions, we next assess the results of these two other reports quantitatively, in the context of our results. In these two studies, $I_{\text{Ca}}$ was expressed relative to the value at the lowest frequency, which was 0.033 Hz in Sanguinetti et al. (1986) and 0.1 Hz in Hadley and Hume (1988); the values below were approximated from the published records, and are renormalized relative to the frequencies indicated. In the study of Sanguinetti et al. (1986; see their Fig. 11), the ratio of $I_{\text{Ca}}$ at 3 Hz to that at 0.5 Hz (at 37°C) was 0.93 (basal) and 0.70 (Bay K 8644), and the 1–0.33 Hz ratio of $I_{\text{Ca}}$ (at 20–22°C) was 0.91 (basal) and 0.83 (Bay K 8644) in the study of Hadley and Hume (1988; see their Fig. 5). These numbers fall into a similar range as ours; at 33–37°C we determined mean ± SEM steady state values of 0.89 ± 0.035 (basal) and 0.79 ± 0.012 (Bay K 8644), which did not differ statistically (see Fig. 6 C). The similarity of the three sources of data cited in this comparison, suggests that the voltage-dependent inhibitory effect of Bay K 8644 on $I_{\text{Ca}}$ only enhances the frequency-induced reduction of $I_{\text{Ca}}$ when the control frequency is extremely low.

Frequency Response of $I_{\text{Ca}}$ in the Presence of IBMX or Bay K 8644

Negative staircases were small in the presence of IBMX or Bay K 8644, despite the 134 and 123% mean elevations of peak $I_{\text{Ca}}$ (at 0.5 Hz) induced by the drugs. Similar increases of $I_{\text{Ca}}$ (at 0.5 Hz) were attained with isoprenaline, forskolin, and CPT-cAMP, but an extra component of frequency-induced inhibition of current was observed. These observations rule out any mechanism for extra inhibition which is solely dependent upon a reduced ionic gradient pursuant to increased Ca$^{2+}$ entry during rapid pulsing of cAMP-enhanced $I_{\text{Ca}}$, because the increase of $I_{\text{Ca}}$ per se did not cause the extra inhibition when it was induced by IBMX or Bay K 8644.

In further support of this assertion, analysis of integrated $I_{\text{Ca}}$ at 0.5 and 3 Hz (basal or with Bay K 8644) showed that large values of Ca$^{2+}$ entry at 0.5 Hz resulted in only moderate reductions of Ca$^{2+}$ entry at 3 Hz. However, similar amounts of Ca$^{2+}$ entry at 0.5 Hz resulted in substantial reductions of $I_{\text{Ca}}$ on switching to 3 Hz, when isoprenaline was present, either alone or in combination with Bay K 8644. Also, the lack of diminution of the extra inhibition after reducing the dialysate
EGTA from 5 to 0.1 mM (see Fig. 3D) suggests that the mechanism is unaffected by an elevation in the background Ca\(^{2+}\) level (as judged by the consistent twitching of the cells with electrical stimulation, and the accelerated inactivation of I\(_{\text{Ca}}\)). Rather, whatever the set point might be for background Ca\(^{2+}\), it seems to be the increase in Ca\(^{2+}\) entry during the 3-Hz staircases that causes a recruitment of the inhibitory process.

Hence, there is a requirement for both cAMP (Fig. 6) and Ca\(^{2+}\) (Fig. 4) in the extra inhibition. While there could be an activating influence of cAMP on the process of I\(_{\text{Ca}}\) reduction, the extra inhibition was not observed when cAMP levels were elevated (cf. Figs. 5 and 6) but phosphodiesterases were inhibited pharmacologically with IBMX (cf. Fig. 5). One interpretation is that high cAMP levels are required in the first place for the phosphodiesterase influence to be exerted, whether or not cAMP is also required to activate (see Beavo and Reifsnyder, 1990) the phosphodiesterase isoenzymes involved. The results are consistent with a recruitment of Ca\(^{2+}\)-dependent phosphodiesterases by rapid stimulation, because the extra inhibition was not observed (a) with isoprenaline applied while recording I\(_{\text{Ca(Na)}}\) in Ca\(^{2+}\)-free bathing solutions (Fig. 4) or (b) with IBMX applied when Na\(^{+}\) (not shown) or Ca\(^{2+}\) (Fig. 5) carried the Ca\(^{2+}\) channel current.

**Influence of Ca\(^{2+}\) Entry on I\(_{\text{Ca}}\)**

When studying the enhancement of I\(_{\text{Ca}}\) by cardiotonic steroids, Marban and Tsien (1982) suggested a facilitatory role of intracellular Ca\(^{2+}\) on I\(_{\text{Ca}}\), possibly mediated through Ca\(^{2+}\)-dependent phosphorylation. Lee, Marban, and Tsien (1985), in the context of a study on inactivation, hypothesized that a process providing positive regulation of Ca\(^{2+}\) channels might be highly Ca\(^{2+}\) sensitive, and a negative regulatory process acting at higher intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]) would cause Ca\(^{2+}\)-dependent inactivation. Fedida, Noble, and Spindler (1988b) came to a similar conclusion after finding that conditions which loaded the cell with Ca\(^{2+}\) greatly reduced the ability of stimulation rate to modify I\(_{\text{Ca}}\), whereas moderate elevations of [Ca\(^{2+}\)]\(_{i}\) (sufficient to enhance contraction) increased the modulation.

In the transition from one stimulation rate to another, there can be a recruitment of and balance between stimulatory and inhibitory Ca\(^{2+}\)-dependent enzymes. Some phosphodiesterases and phosphatases play roles in the modulation of membrane currents (e.g., Chad and Eckert, 1986; Luk'yanets, 1992), and adenylyl cyclase in the heart can also be influenced by Ca\(^{2+}\) (see Cooper and Brooker, 1993). Through their influences on cAMP levels and phosphorylation of Ca\(^{2+}\) channels, these Ca\(^{2+}\)-sensitive modulatory mechanisms would result in feedback control of I\(_{\text{Ca}}\). The role of Ca\(^{2+}\) release from the sarcoplasmic reticulum was not investigated. However, the frequency response of I\(_{\text{Ca}}\) in both the basal state and with isoprenaline was unchanged when the dialysate Ca\(^{2+}\) was buffered with 0.1 mM rather than 5 mM EGTA (see Fig. 3D). Also, using fura-2, we were unable to resolve Ca\(^{2+}\) transients in the presence of EGTA concentrations >5 mM in the dialysate (Pelzer, Kasper, You, Foley, and Pelzer, 1995). Nevertheless, even the abolition of cytoplasmic Ca\(^{2+}\) transients by high EGTA does not completely rule out a role of Ca\(^{2+}\) release on I\(_{\text{Ca}}\) because of privileged communication between L-type Ca\(^{2+}\) channels and ryanodine receptors (Sham, Cleemann, and Morad, 1995).
The results presented argue for an enzymatic component in the frequency-induced reduction of $I_{\text{Ca}}$. This component was evident when cAMP levels were elevated, but only if phosphodiesterases were not inhibited. Hence, the extra inhibition requires an elevation of cAMP, and the accompanying elevation of $I_{\text{Ca}}$ (i.e., increased $\text{Ca}^{2+}$ entry) is not itself sufficient to cause the effect (see Fig. 6). However, two possible explanations remain. Firstly, the extra component of frequency-induced inhibition of $I_{\text{Ca}}$ could result from reduced $\text{Ca}^{2+}$ channel phosphorylation after a reduction in the activity of cAMP-dependent protein kinase (PKA), pursuant to phosphodiesterase activation and cAMP degradation. Secondly, the effect could arise from an increased susceptibility of $\text{Ca}^{2+}$ channels to inactivating influences. While the former paradigm is the most direct interpretation, the latter could theoretically be mediated either by allosteric modifications of phosphorylated channels, or by some other influence of cAMP on $\text{Ca}^{2+}$-dependent inactivation, which would also make the effect susceptible to phosphodiesterase inhibition. Interpretation (b) is less likely because $\text{Ca}^{2+}$ channels would be phosphorylated in the presence of either IBMX or isoprenaline, yet the response of $I_{\text{Ca}}$ in the presence of IBMX was markedly different from its response in the presence of isoprenaline.

In the context of interpretation (a), there is an interesting dichotomy posed by a comparison of Figs. 7 and 8. On one hand, applying isoprenaline while evoking $I_{\text{Ca}}$ at 1–3 Hz caused a large increase in $I_{\text{Ca}}$, with a disproportionately large restitution on relieving the stimulation to 0.5 Hz. On the other hand, after elevating $I_{\text{Ca}}$ severalfold by applying isoprenaline, forskolin, or CPT-cAMP at 0.5 Hz, a subsequent increase in stimulation frequency sometimes caused inhibition that was so large as to reduce $I_{\text{Ca}}$ below its basal value at the same frequency. The effect might indicate that in the latter protocol, the recruitment of inhibitory processes can reduce a component of basal $\text{Ca}^{2+}$ channel current (or of basal phosphorylation of the channel proteins), as well as reducing the up-modulated component which was shown (Fig. 5) to be entirely susceptible to IBMX.

**Functional Consequences of the Findings**

Despite the large reduction of $I_{\text{Ca}}$ with rapid stimulation in the presence of high concentrations of isoprenaline or forskolin, the absolute amount of $\text{Ca}^{2+}$ entry is still enhanced. This was the case both because (a) $I_{\text{Ca}}$ brings $\text{Ca}^{2+}$ into the cell more often, and (b) the $\text{Ca}^{2+}$ entry per pulse is not always less than basal current at the same frequency (cf. Fig. 8 as a rough indicator of the second factor).

The more frequent entry of $\text{Ca}^{2+}$ is at least as important as the increment of $I_{\text{Ca}}$ in the myocardium with isoprenaline, because the latter is mitigated by extra inhibition at high frequencies. For example, with a 7.5-fold increase in frequency from 0.08–0.6 Hz, we recorded a 20% decrease in $I_{\text{Ca}}$ concomitant with a 45% net increase in $[\text{Ca}^{2+}]$, from 190 to 275 nM, in the presence of 0.2 mM intracellular EGTA (Pelzer et al., 1995). Conversely, indo-1 measurements in the working heart (stimulated at a fixed rate) indicate that functionally important changes in $[\text{Ca}^{2+}]$, transients can be fairly subtle in magnitude after applying 1 µM isoprenaline (Wikman-Coffelt, Wu, and Parmley, 1991). Hence, the degree of enhancement of $\text{Ca}^{2+}$ entry, induced by cAMP, depends upon the stimulation rate, and $I_{\text{Ca}}$ at lower heart rates would be expected to increase to a greater extent than $I_{\text{Ca}}$ at elevated heart.
rates. This design would allow greater responsiveness of $I_{Ca}$ to increased cAMP levels at low heart rates, while setting limits on elevations in Ca$^{2+}$ entry at higher frequencies in order to prevent Ca$^{2+}$ overload.

It is concluded that Ca$^{2+}$-dependent (and possibly other) phosphodiesterases constitute an important modulatory system for the Ca$^{2+}$ and cAMP messenger systems in ventricular heart cells, the greatest influence being attained when the heart rate increases. The negative feedback would safeguard against Ca$^{2+}$ overloading, and may serve to limit the positive effects of stimulatory processes such as $I_{Ca}$ facilitation and/or Ca$^{2+}$ channel phosphorylation by kinases.

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