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ACCELERATED COMMUNICATION

Dihydropyridine Bay K 8644 Activates T Lymphocyte Calcium-Permeable Channels

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SUMMARY

The effects of the dihydropyridine calcium channel agonist Bay K 8644 on indo-1-loaded Jurkat human leukemia T lymphocytes was assessed by flow cytometry. Bay K 8644 from 10^{-9} to 10^{-4} M caused a dose-dependent rise in the intracellular free Ca concentration, an effect that was not mimicked by the dihydropyridine Ca antagonist nifedipine. Single channel recordings by the extracellular patch-clamp technique indicated that Bay K 8644 activated an 8-pS, barium-permeable channel that opened as bursts of brief events. The channel appeared to be identical to the previously described voltage-insensitive, messenger-mediated, calcium-permeable channel involved in T cell activation. The predominant effect of Bay K 8644 on these channels was to increase the probability of channel reopening, apparently without a major effect on mean channel open-time. The results suggest that the dihydropyridine Ca agonist Bay K 8644 interacts with both voltage-gated and receptor-operated Ca channels and also suggest potential strategies for development of a new class of immunomodulatory drugs.

The dihydropyridine calcium channel blockers, including nifedipine, are the most potent and specific of all the Ca channel antagonists that have been investigated (1, 2). Single channel studies have shown that these drugs block current flow through voltage-gated, L-type Ca channels (3). A small modification of the nifedipine molecule produces dihydropyridine agonists, including Bay K 8644, whose primary effect is to increase current flow through the voltage-gated, L-type Ca channel (4–7). The amino acid sequence of one subunit of the dihydropyridine receptor of rabbit skeletal muscle was recently predicted by cloning and sequencing analysis of the cDNA (8). By revealing structural and sequence similarities to the voltage-dependent sodium channel, it provided strong evidence that the dihydropyridine receptor actually resides within the Ca channel macromolecule itself.

The effects of dihydropyridine antagonists and agonists on voltage-sensitive Ca channels in a wide variety of tissues have been described (9–15). We report here a new action of the calcium agonist Bay K 8644 on the voltage-insensitive, messenger-mediated calcium-permeable channel of the Jurkat T lymphocyte cell line. Several investigators have documented the lack of voltage-gated Ca channels in T lymphocytes (16–18). Instead, these cells have been shown to have low conductance, calcium- and barium-permeable channels (8), which, via the mediation of the second messenger inositol 1,4,5-trisphosphate (19), are responsible for calcium influx during mitogen activation. This channel has been classified in a new group of so-called receptor-operated calcium channels (20).

It has previously been shown that the dihydropyridine antagonists inhibit, in a dose-dependent fashion, mitogen-induced 45-calcium influx in T lymphocytes (21). Because, however, 2.4×10^{-5} M nifedipine was needed for 50% suppression of T cell proliferation, it was unclear whether nifedipine was serving as a specific or a nonspecific Ca antagonist. We report here that Bay K 8644 increases intracellular calcium in nonstimulated T cells. Single channel recordings suggest that this action occurs through a direct effect of Bay K 8644 on the T cell Ca-permeable channel to increase channel opening probability. This suggests that dihydropyridines do directly modulate current flow through the calcium-permeable channel of the T lymphocyte and opens a new line of investigation for the development of related but higher potency drugs that may modify immune function.

Materials and Methods

Cells. All experiments were performed on cells of the malignant T lymphocyte cell line Jurkat-E6-1, which were maintained in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum, 50 μg ml^{-1} gentamycin, and 2 mM L-glutamine, at 37° in humidified 5% CO_2 atmosphere.

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ABBREVIATION: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Reagents. Bay K 8644 was the generous gift of Dr. Alexander Scriabine, Director of the Institute for Preclinical Pharmacology, Miles Laboratories, Inc. (New Haven, CT). Indo-1 was purchased from Molecular Probes (Eugene, OR).

Indo-1 loading. All loading procedures were performed in a dark area. Before loading, the cell suspension was incubated with 1 μM indo-1 at 37° for 45 min. The cells were washed and resuspended twice with fresh RPMI 1640 medium and maintained in the dark at room temperature (20–22°) until experimentation began.

Flow cytometry. A total of 0.3 ml of indo-1-loaded cell suspension was studied at room temperature, using a FACS IV flow cytometer (Becton-Dickinson Co., Mountain View, CA). Flow rate was kept between 300–700 cells/sec. UV excitation at 350 nm was made and emissions at 480 nm (blue) and 405 nm (violet) were recorded. In every run, after a control period of 30 sec of baseline data was recorded, the cells were removed from the cytometer in order to add the test reagent. After vortex mixing, the tube of cells was rapidly returned to the instrument, and data sets were recorded for 500 sec. In addition to the blue and violet fluorescence, the forward scatter signals were routinely recorded. The ratio of violet to blue fluorescence was calculated using the Consort 40 software analysis program (Becton-Dickinson Company, Mountain View, CA).

Extracellular patch clamp recording. Jurkat cells were washed twice and resuspended in the solution outlined in the legend to Fig. 2. All experiments were performed at room temperature (20–22°). Single-channel currents were recorded in the cell-attached recording configuration of the extracellular patch clamp technique (22) with a List-EPC7 patch-clamp amplifier. The Sylgaard-coated patch pipettes were pulled from thick-walled borosilicate glass pipettes to a lumen diameter of 0.5 μm. Current signals were stored on videocassettes and later digitized for analysis on a microcomputer (IBM PC-AT).

Methods of data analysis. Software analysis programs written by William Anderson, P.E. (Engineering Software Services, Palo Alto, CA) were used for the generation of average current amplitudes (Fig. 3B) and power spectral densities (Fig. 4). To determine average current amplitude (I), records were filtered at 2 kHz, sampled at 10 kHz, and fed into the computer. For any given current record, a value for baseline current was first determined by the method of maximum number of zero crossings, a nonparametric measure not biased by outliers. Average current was computed by integrating the current signal and dividing by total time, using the trapezoidal rule. The baseline determination was then subtracted from this value to obtain average current amplitude (I). The power spectral density functions were calculated from currents in the absence of and in the presence of agonist. Records were filtered at 2 kHz, digitized at 4 kHz, and fed into the computer. The fast Fourier transform (F.F.T. algorithm), which yielded a two-sided spectral density function, was calculated for eight 4096-point segments, which were then averaged point by point. The time constants (τ) for each spectra were calculated according to the following equation: $\tau = 1/2\pi f_c$. The single channel conductance γ is estimated from the following equation:

$$\gamma = \frac{S_0\ \pi\ f_c}{2I\ (V_M - V_{eq})}$$

where S_0 = zero frequency asymptote of the spectral density; f_c = cut-off frequency; I = mean current amplitude; V_M = the patch membrane potential; V_{eq} = the zero current potential.

In order to determine single channel amplitude, records were filtered at 2 kHz and digitized at 10 kHz. The automated single channel analysis program of Sachs *et al.* (23), implemented by C. Lingle (Washington University, St. Louis, MO), was used to provide an estimate of single channel amplitudes cited in the text. The baseline level (corresponding to 0 pA) was located using an algorithm that maximizes the number of crossings through a horizontal line. An event detection threshold was set at 0.5 times the predetermined single channel amplitude (by eye, which was slightly higher than computer-derived mean secondary to inclusion by computer of short events that do not reach full amplitude), whereas multiple event exclusion threshold was set at 1.5 times the predetermined single-channel amplitude. Estimate of mean single channel amplitude was obtained from the gaussian fit of the amplitude histogram of detected openings.

Results

Effect of Bay K 8644 on intracellular free calcium of Jurkat cells assessed by indo-1 and flow cytometry. Relative intracellular free calcium concentrations of large numbers of cells can be analyzed with the use of flow cytometry and the recently described Ca fluorescent indicator dye, indo-1 (24). Indo-1 exhibits large changes in fluorescence emission wavelength upon calcium binding. Use of the ratio of intensities at the two emission wavelengths (480 nm, blue, and 405 nm, violet) gives a relative indication of intracellular calcium concentration, independent of variability in intracellular dye concentration. Thus, a rise in intracellular free calcium is indicated by an increase in the violet:blue fluorescence ratio. When indo-1-loaded nonstimulated Jurkat T cells are exposed to increasing concentrations of Bay K 8644, a small but reproducible increase in the mean violet:blue fluorescence ratio, indicating a concentration-dependent increase in cytosolic free Ca, was seen (Fig. 1). The EC_{50} on a series of six similar experiments was estimated to be 10^{-8} M. Bay K 8644 has been reported to be a partial agonist for voltage-gated Ca channels (5) with antagonistic actions predominating at higher Bay K 8644 concentra-

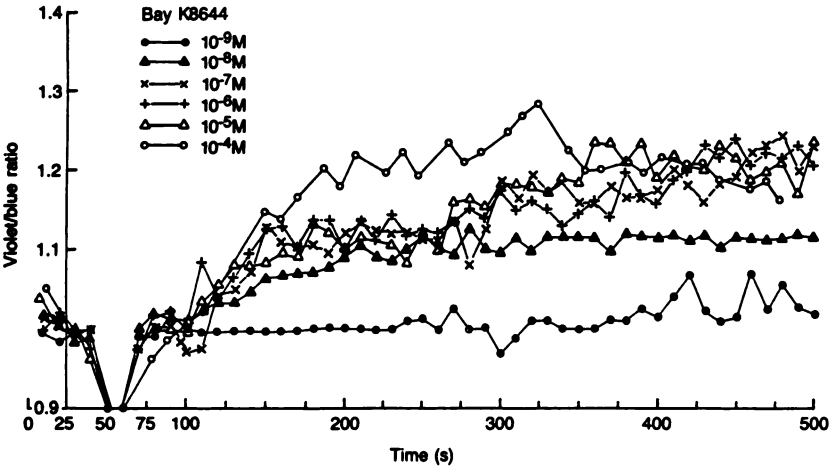

Fig. 1. Flow cytometric analysis of mean indo-1 violet:blue fluorescence ratio as a function of time and Bay K 8644 concentration. Bay K 8644 was added in the indicated concentration to indo-1-loaded Jurkat cells at time 25 sec (coinciding with the vertical axis). The mean violet:blue ratio is normalized to 1.0 for cells immediately before Bay K 8644 addition. Transient change seen during the 40 sec after addition of Bay K 8644 are artifacts due to cessation of flow.

tions if the cell membrane is depolarized (25). No evidence for partial agonism with antagonism at higher concentrations was seen in any of our experiments on T lymphocytes, however.

In data not shown, indo-1-loaded Jurkat cells exposed to similar concentrations of nifedipine, the dihydropyridine Ca channel antagonist, did not show evidence of drug-induced increase in intracellular free Ca. Nifedipine at high concentrations (greater than or equal to 10^{-5} M) does partially block increases in intracellular free Ca in mitogen-stimulated T cells, however. The latter results are in agreement with the published results of other investigators, including Birx *et al.* (21), who reported dose-dependent suppression of phytohemagglutinine-stimulated Ca uptake and proliferation of T lymphocytes by nifedipine. The high concentrations of Ca antagonist needed to suppress Ca uptake may argue for a nonspecific action of dihydropyridine on Ca uptake and cell proliferation. On the other hand, the opposing actions of the structurally similar compounds, Bay K 8644 and nifedipine, on T cell Ca uptake are consistent with the interpretation that the dihydropyridines have a specific effect on the T cell calcium-permeable channel as an agonist and antagonist, respectively.

Single channel recordings of the effect of Bay K 8644 on the T cell calcium-permeable channels. To further investigate the molecular mechanism of the observed Bay K 8644 effect on Ca accumulation, single channel recordings of Jurkat cell-attached patches were made before and after exposure of the cell to Bay K 8644. In general, the following recording conditions were used. The pipette contained an isotonic $BaCl_2$ solution, both to maximize the current flowing through the Ca-permeable channels and to block outward K^+ currents, which are prevalent in these patches (16, 17). The cells were suspended in a low calcium, high extracellular K^+ solution for the following reasons. A nominal 10^{-7} M external Ca concentration was chosen to eliminate the possibility that a rise in cytosolic free Ca secondary to Bay K 8644 is the actual cause of observed conductance changes in the cell-attached patch. It is well documented that Ca ions are common second messengers both to activate some calcium-permeable channels (26) and to inactivate others, including the voltage-gated Ca channels (27) and the T cell Ca-permeable channel (18). External isotonic KCl was employed to zero the resting membrane potential of the cells. By ensuring absolute rather than relative voltage determinations, we were best able to maximize the separation of the channel of interest from a prevalent second channel in the patch with similar appearance but different ionic permeability. Whereas the Ca-permeable channels invariably reverse at greater than +20 mV membrane potential under these recording conditions, indicating relatively greater divalent cation permeability, the second channel reversed at approximately −10 mV and was presumed to be a chloride-selective channel. At depolarized or hyperpolarized voltages, the two current types became difficult to distinguish, but complete and reliable separation of currents was ensured by recording at 0 mV, at which currents flowing through the Ca-permeable channel are inwardly directed and currents flowing through the second channel are outwardly directed (see, for example, control record, Fig. 2B). Finally, Bay K 8644 was added to the bathing solution. As in previous patch-clamp studies with this compound, the highly lipophilic dihydropyridine derivative is assumed to have access to the channel in the cell-attached patch through the lipid phase of the membrane, because no drug was present in the pipette, and the pipette-membrane seal represents a virtually absolute diffusion barrier.

Fig. 2 shows the effect of Bay K 8644 (50 μM) on unitary currents recorded from a cell-attached membrane patch. Trace A is a trace at slow sweep speed measured from the same patch immediately before and after Bay K 8644 was added to the bath. Before drug addition, intermittent outward currents, presumed to be flowing through the chloride channel of the type described above, were seen; no inward currents were recorded during several minutes of observation before drug addition. Within seconds after adding Bay K 8644 to the bath, inward currents were elicited in the membrane patch. Because Ba is the only ion with an inwardly directed driving force at this membrane potential and under these ionic conditions, we assume that the Bay K 8644-induced inward currents are carried by Ba through divalent cation-permeable channels. No measureable effect on the outward current was detected. The control

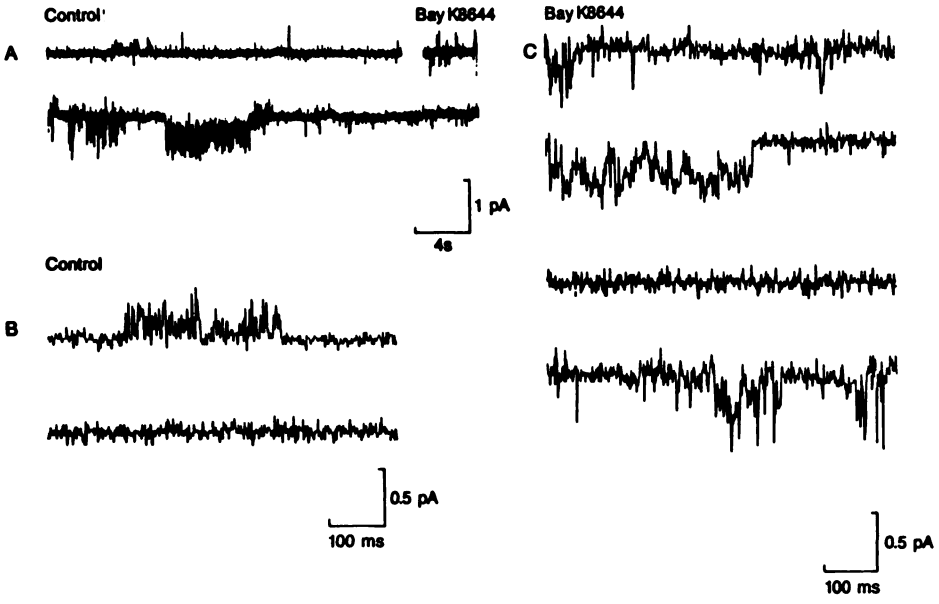

Fig. 2. Single channel recordings of Bay K 8644-induced inward Ba currents. The patch pipette contained 110 mM $BaCl_2$, and 10 mM HEPES-KOH (pH 7.3). The bath solution contained (in mM): 150 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES-KOH (pH 7.3). Holding potential was zero mV. Currents were filtered at 1 kHz (−3 dB). Trace A shows consecutive records from a membrane patch under control conditions and after a solution containing Bay K 8644 (50 μM) was bath perfused (coinciding with the 1.5-sec interruption in the current trace). Outward currents are seen as upward deflections and inward currents are seen as downward deflections. Inward Ba currents were seen only after drug addition. Trace B shows a portion of the control and trace C shows a portion of the Bay K 8644 traces at higher gain and faster sweep speed.

and Bay K 8644 traces are shown at higher gain and sweep speed in Fig. 2, B and C. In the control trace, only the previously described outward currents were seen. Low amplitude inward Ba currents were seen within seconds after adding Bay K 8644 to the bath, however. Bay K 8644-induced currents flowed through a channel of low conductance (7.6 ± 0.5 pS, mean ± SD, eight experiments, assessed by noise analysis as described in Materials and Methods) and positive reversal potential (greater than +20 mV in the 28 patches analyzed). Because the conductance, reversal potential, and kinetic appearance of bursts of brief openings that typified the Bay K 8644-activated channel are virtually identical to the properties previously reported for T cell calcium-permeable channels (18), we assume that the Bay K 8644-activated channel and the calcium-permeable channel are the same channel.

In the example patch illustrated in Fig. 2, the inward Ba currents appeared only after addition of the drug to the bath, raising the possibility that Bay K 8644 activated previously unavailable channels. In other patches, however, Bay K 8644 appeared to simply increase the frequency of channel opening relative to control recordings. A representative patch with inward Ba currents in control, which substantially increased in frequency after Bay K 8644 addition, is seen in Fig. 3A. In order to quantify the effect of Bay K 8644 on the average Ba current flowing through the T cell permeable channel, we integrated inward currents in patches before and after drug addition (see Materials and Methods). In six of nine patches thus analyzed, Bay K 8644 increased the average inward Ba current, either by inducing or increasing the frequency of channel openings. (Three of the patches had no apparent channel openings, either before or after Bay K 8644.) Average current (I) is directly related to channel opening probability (p), according to the equation $I = npi$, where n equals the number of channels in a patch and i equals single channel amplitude. Because no discernible effect was seen on single channel amplitude (i) [e.g., in the patch in Fig. 3A, single-channel amplitude estimates derived from 10-sec records of control were 0.41 ± 0.11 pA (mean ± SD, 1014 determinations) and of Bay K 8644 were 0.39 ± 0.12 (611 determinations)] we concluded that Bay K 8644 increases the Ba current by making channels available for opening (n) and/or by increasing the probability (p) that previously available channels are open (the latter two possibilities are difficult or impossible to distinguish in single channel recordings).

Noise analysis of Bay K 8644-augmented barium currents. At the single channel level, the most consistent effect of Bay K 8644 on voltage-gated Ca channels has been to cause an increase in the mean open time (5–7). Alternatively, Brown *et al.* (28) reported that the major effect of Bay K 8644 on voltage-gated calcium channels of guinea pig ventricular myocytes was to increase the probability of channel reopening (seen as an increase in the number of openings per sample), with an increase in the number of available channels also considered. Prolongation of open time was only seen at higher Bay K 8644 concentrations, on the order of 10^{-5} M.

A drug-induced increase in number of openings per unit time can easily be discerned by eye from our recordings. In addition, we intermittently observed "long" openings in our single channel recordings of Bay K 8644-induced inward Ba currents (see Fig. 2C, *second trace*). In order to determine whether prolongation of mean open time underlies the increased average channel current secondary to the drug, we assessed the Bay K 8644 effect on channel gating kinetics by the technique of noise analysis. Noise analysis was chosen over standard single channel interval analysis because the inherently low signal-to-noise ratio, the rapidity of channel openings relative to recording bandwidth, and the almost invariable presence of multiple channels within a patch generally make interval analysis unsuitable.

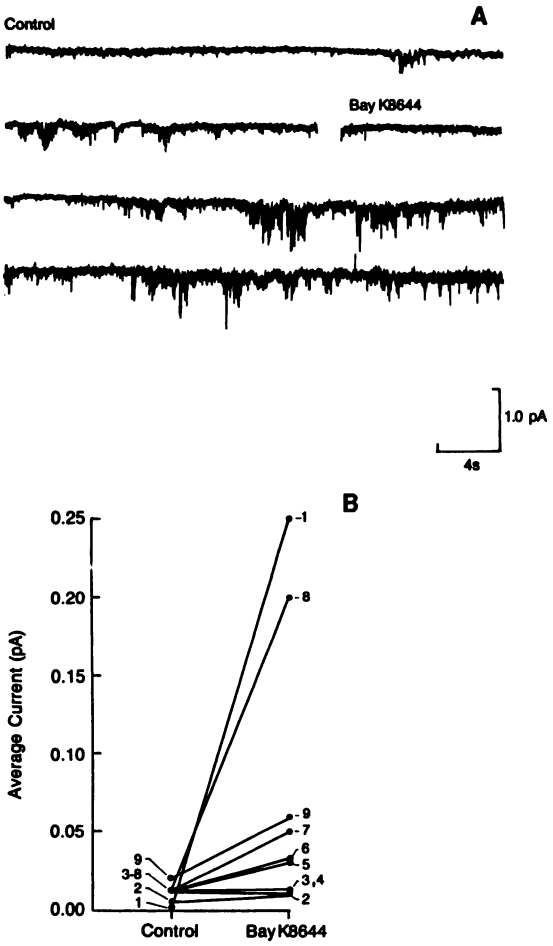

Fig. 3. Effect of Bay K 8644 on average inward Ba current. A. Single channel recordings of Bay K 8644 augmented inward Ba currents in a cell-attached patch. Recording conditions were the same as in the legend to Fig. 2. Inward Ba currents (downward deflections) were present in control conditions; after addition of drug an increase in channel-opening frequency was readily discernible by eye. B. Average inward Ba current before and after Bay K 8644 addition. Currents from nine different cell-attached patches were integrated (as outlined in Materials and Methods) for a representative 8-sec period before and after Bay K 8644 (50–100 μM) was added to the bath.

The power spectral densities of single channel current fluctuations before and after drug addition were compared. In those patches without channel openings before drug exposure, no lorentzian component could be discerned in the spectral density of the current fluctuations. However, in those patches in which openings were observed both before and after drug addition (as in Fig. 3A), the spectrum of Bay K 8644-induced current fluctuations have the same form as the control spectrum (Fig. 4). In all cases, the Ca-permeable channel current fluctuations have a power spectrum with two (1/frequency^2) components, indicating a complex kinetic model for channel operation. The duration of time constants appears relatively uninfluenced by

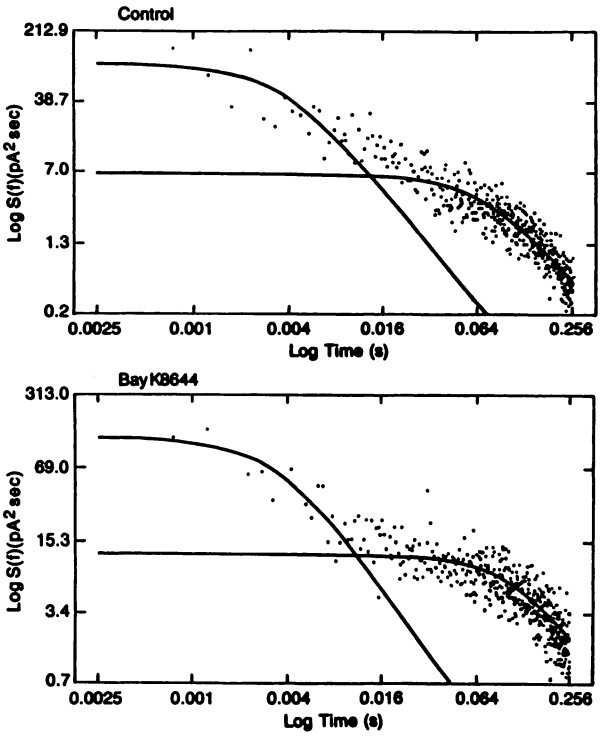

Fig. 4. Effect of Bay K 8644 on the fluctuation spectra of single channel currents recorded from a cell-attached patch. Recording conditions were identical to those in the legend to Fig. 2. Each of the spectrum presented is an average of eight spectra derived from sequential 1-sec records (sampling interval was 250 μsec; records were filtered at 2 kHz). Spectra are in logarithmic coordinates. The *solid lines* are the lorentzian fit to the spectra; in each case the spectrum is best described by a two-component fit. The long and short time constants corresponding to the cut-off frequency were 5.8 and 0.3 msec in the control recording and 6.0 and 0.2 msec in the Bay K 8644 recording.

the action of Bay K 8644. From the similarity of the spectra in control and drug-induced records, we conclude that it is unlikely that Bay K 8644 is increasing average current by prolonging mean open time, as in voltage-gated channels. The major Bay K 8644 effect must, therefore, be on channel opening or reopening probability, yielding an increase in the number of channel openings per unit time.

Discussion

The data presented herein are most consistent with the conclusion that Bay K 8644 exerts a direct agonist action in the T lymphocyte calcium-permeable channel, one of a newly defined class of receptor-operated Ca channels. There are several sites of differing affinity for the dihydropyridines (29). These drugs bind to voltage-gated Ca channels with high affinity and stereospecifity in a number of tissues (30). Low affinity binding sites may also be associated with the voltage-gated Ca channels. The characteristics of use dependency (31, 32) and enhanced inhibition of Ca current at depolarized membrane potential (13, 25) strongly argue for state-dependent binding of the dihydropyridines to the voltage-gated Ca channel, with variable affinity for the resting closed state, the open state, and the inactivated state of the voltage-gated Ca channel. There is other evidence that links the low affinity dihydropyridine binding sites to adenosine receptors in rat brain (33) and human red blood cell (34). The finding reported herein, that Bay K 8644 exerts a direct agonist action on the calcium-permeable channel of T cells, may provide evidence for another type of Bay K 8644 low affinity binding site, i.e., receptor-operated Ca channels. The finding that Bay K 8644 is an agonist for both voltage-gated and receptor-operated Ca channels raises the possibility that the two Ca channel types evolved from a common evolutionary origin, bifurcating with the development of alternative gating strategies.

This is the first step in the characterization of the pharmacological profile of the T lymphocyte calcium-permeable channel, which is critical to cellular activation and division. Bay K 8644 exerts its effect on this channel in a concentration range that makes the observations more of experimental rather than practical interest. Nevertheless, the documentation of a class of drugs that influence T lymphocyte Ca accumulation may provide new developmental strategies for drugs that affect immune function.

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