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Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells

(Ca-channel blockers/Ca-channel modulation/single-channels/patch-clamp/heart)

S. KOKUBUN AND H. REUTER*

Department of Pharmacology, University of Berne, CH-3010 Berne, Switzerland

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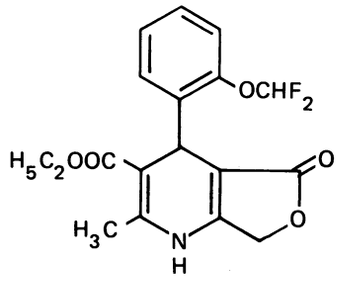
ABSTRACT **The dihydropyridine derivatives CGP 28392 and BAY K 8644 exert a strong positive inotropic effect in mammalian cardiac muscle, presumably by increasing Ca influx during the action potential. Analysis of the drug effects at the level of single Ca channels by means of the patch-clamp method revealed complicated changes in the kinetics of channel gating. The prevailing effect is a prolongation of the mean open time of Ca channels. In addition, the intervals between channel openings can be slightly prolonged. Ensemble averages of single-channel traces showed a concentration-dependent increase in the mean current amplitude by the drugs. In contrast to β-adrenoceptor agonists, the increase in Ca current by the dihydropyridine derivatives is not associated with an increase in the intracellular cyclic AMP level.**

As a relatively new class of drugs, dihydropyridine derivatives have gained considerable interest both therapeutically and experimentally. Nifedipine and nitrendipine are Ca-channel blockers (1, 2) widely used in antihypertensive therapy. These drugs were also the first to be used as radioligands in an attempt to label and characterize Ca channels (3–5). Recently, however, two new drugs of this class have been synthesized that have been proposed to increase rather than to decrease the Ca permeability of excitable cells (6, 7). We have investigated the effects of these drugs [CGP 28392 (6) and BAY K 8644 (7); see structures I and II, respectively] on single Ca channels in cultured cardiac cells by means of the patch-clamp method (8). We have found that both drugs modify the voltage-dependent gating of Ca channels and thereby prolong the open state of these channels.

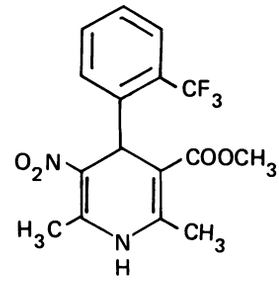
METHODS

Primary cultures of cardiac cells were prepared from hearts of neonatal rats as described elsewhere (9, 10). Myocardial cells were seeded on coverslips at a low density ($\approx 10^5$ cells per ml). Well-differentiated spindle-like cardiac cells showing cross-striations were used 1–3 days after seeding for the electrophysiological measurements.

Single Ca-channel recordings were made with the patch-clamp method (8, 11). The holding potential of the patch was identical to resting potential, which is around −70 mV in these cells as estimated from occasional break-through of the patch. The recording pipette contained 96 mM $BaCl_2$ solution with 20 μM tetrodotoxin, buffered to pH 7.4 with 10 mM Hepes. Single-channel currents, with Ba^{2+} as charge carriers, were measured from cell-attached membrane patches with seal resistances between 10 and 100 GΩ. The cells were superfused with saline of the following composition in mM: NaCl, 137; KCl, 5.4; $MgCl_2$, 2; $CaCl_2$, 0.02; glucose, 10; Hepes buffer, 10 (pH 7.4; temperature, 20.5–21.5°C). Freshly dissolved drugs (CGP 28392 and BAY K 8644 dissolved in 96% ethanol) were added to the bathing solution after a 10- to 15-min control period, during which single-channel recordings were stable. The final ethanol concentration of at most 0.1% did not affect single-channel currents. The drugs were allowed to equilibrate for 6–10 min before the recordings were resumed. Current traces were filtered at 1 kHz. Digitized records (sampling interval, 0.2 ms) were further analyzed by means of a minicomputer (PDP 11/04). From leak-subtracted current traces, we have determined (*i*) the single-channel current designated *i*, (*ii*) the opening probability designated p_o, and (*iii*) the number of channels designated *N*. From many current traces in each experimental condition, we have obtained average currents (*I*) and open-time and closed-time histograms.



Structure I



Structure II

RESULTS

Fig. 1 shows the effect of a high concentration (5 μM) of CGP 28392 on a single Ca channel recorded in a cell-attached membrane patch. The upper five records are consecutive traces measured under control conditions (Fig. 1 *Left*) and in the presence of the drug (Fig. 1 *Right*). The bottom current records are ensemble averages of single-channel traces. The average current was approximately twice as large in the presence of CGP 28392 as in its absence. This effect is primarily due to a large prolongation of the mean open time of the channel. This is obvious from a comparison of the upper five traces in Fig. 1. Whereas in the control records the Ca channel showed the characteristic burst behavior (11, 12), CGP 28392 produced much longer channel openings with only few and brief interruptions. There were periods during consecutive depolarizations in which Ca channels failed to open (11, 12) (nulls; not shown). The number of nulls was not consistently affected by the drug. However, in all experiments with CGP 28392, there was a 10–30% increase in the single-channel current. This is illustrated in Fig. 2 *Upper*. The larger single-channel currents in the presence of the drug were probably not only artifacts caused by better resolution of the long openings of the channel because occasional long openings in the controls also showed smaller current levels. However, small changes in membrane potential and/

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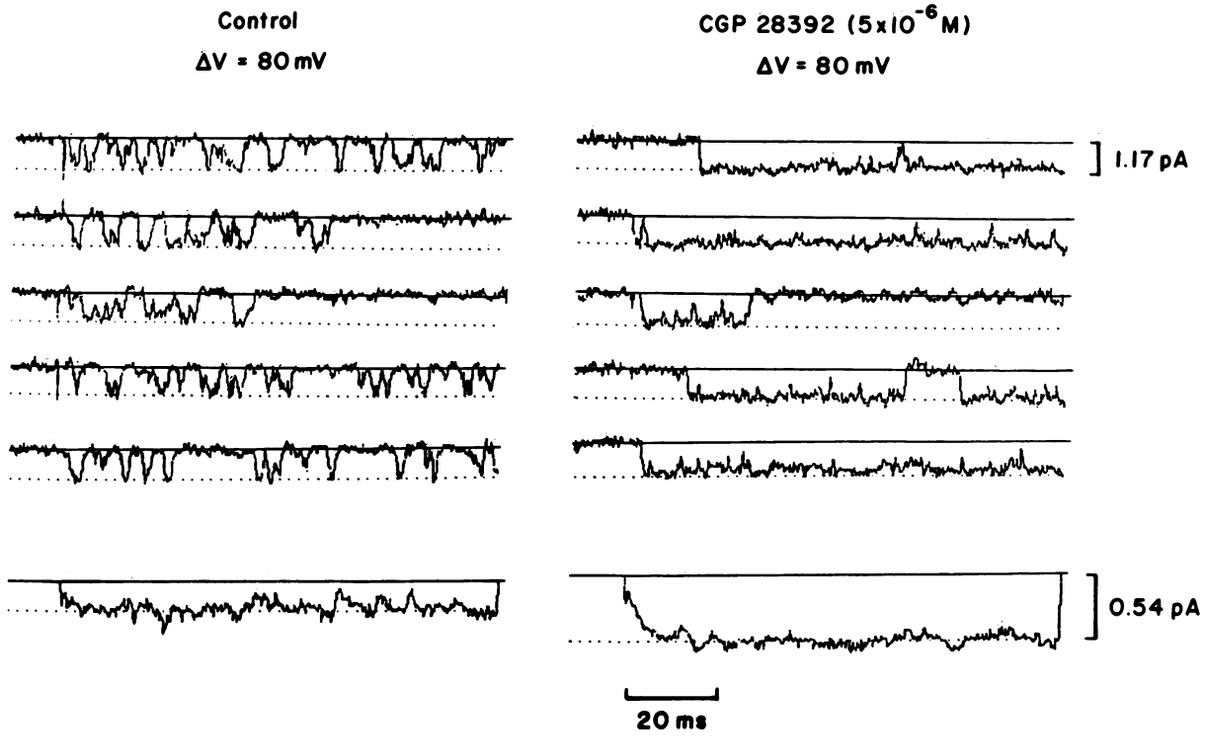

FIG. 1. Recordings of single Ca-channel currents from a cultured cardiac cell in the absence (control) (*Left*) and presence (*Right*) of CGP 28392 (5 μM). The upper five records show consecutive current traces with Ca-channel activity in both conditions. The bottom traces show the average of 39 (control) and 76 (CGP 28392) single-channel current traces, respectively. Heavy lines indicate closed states, and dotted lines indicate open states (fitted by eye) of the Ca channel during 80-mV depolarizing clamp steps of 100-msec duration. The single-channel current amplitudes were on the average 1.12 pA (controls) and 1.20 pA (CGP 28392), respectively.

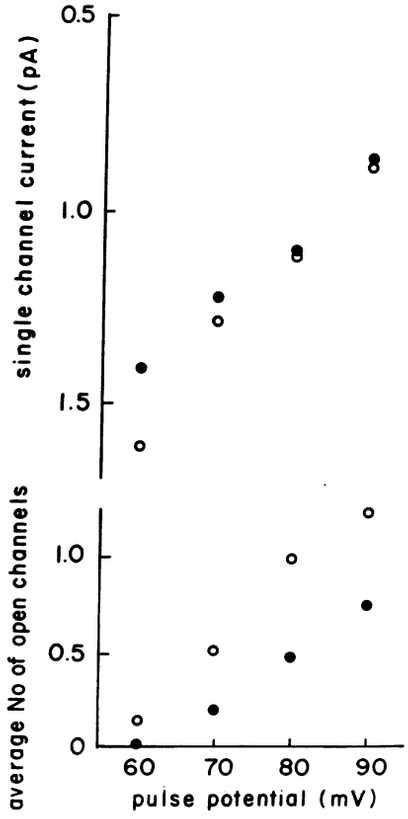

FIG. 2. Voltage dependences of open-channel current (*Upper*) and of open-channel probability ($N \cdot p_o$) (*Lower*) in the absence (●) and presence (○) of CGP 28392 (5 μM). Pulse potentials (abscissa) refer to depolarizing clamp steps from the resting potential of the cell.

or voltage shifts of channel activation could not be excluded. Over a limited voltage range, the single-channel slope conductance increased from 18.3 pS to 24.0 pS in the experiment in Fig. 2. In five experiments, the slope conductance increased from 20.9 ± 1.1 (controls; mean ± SEM) to 23.8 ± 0.9 (CGP 28392) over a 30- to 40-mV voltage range.

Fig. 2 *Lower* shows a considerable increase in the average number of open channels ($N \cdot p_o$) by CGP 28392. This was calculated from $I = N \cdot p_o \cdot i$, where I is the time-averaged current flowing through open channels during the clamp pulses of 100-msec duration, i is the single-channel current, N is the maximum number of single-channel current levels observed in the patch, and p_o is the opening probability of the channels. The increase in the average number of open channels results from an increase in p_o and not in N because the same maximum number of current levels was observed in the controls and in the presence of CGP 28392. We obtained very similar results in seven experiments with the compound BAY K 8644, which also increased the number of current sweeps with long channel openings in a dose-dependent manner, though at approximately 1/10th the concentration as with CGP 28392. Single-channel current was also increased slightly by 4–20% in all experiments, depending on the drug concentration. None of the drugs produced spontaneous Ca-channel openings without depolarizing clamp pulses.

Since the most dramatic effect of both drugs was a marked prolongation of the mean open times of Ca channels, we analyzed the kinetics of single-channel currents in greater detail. Fig. 3 shows histogram analyses of several hundred current traces from one experiment at a single-pulse potential (ΔV = 80 mV). The upper histograms show the distribution of open times of a single Ca channel without and with CGP 28392 (≈1 μM) present in the bathing solution. This drug concentration was probably below the saturating level. Although under control conditions open times were exponentially distributed

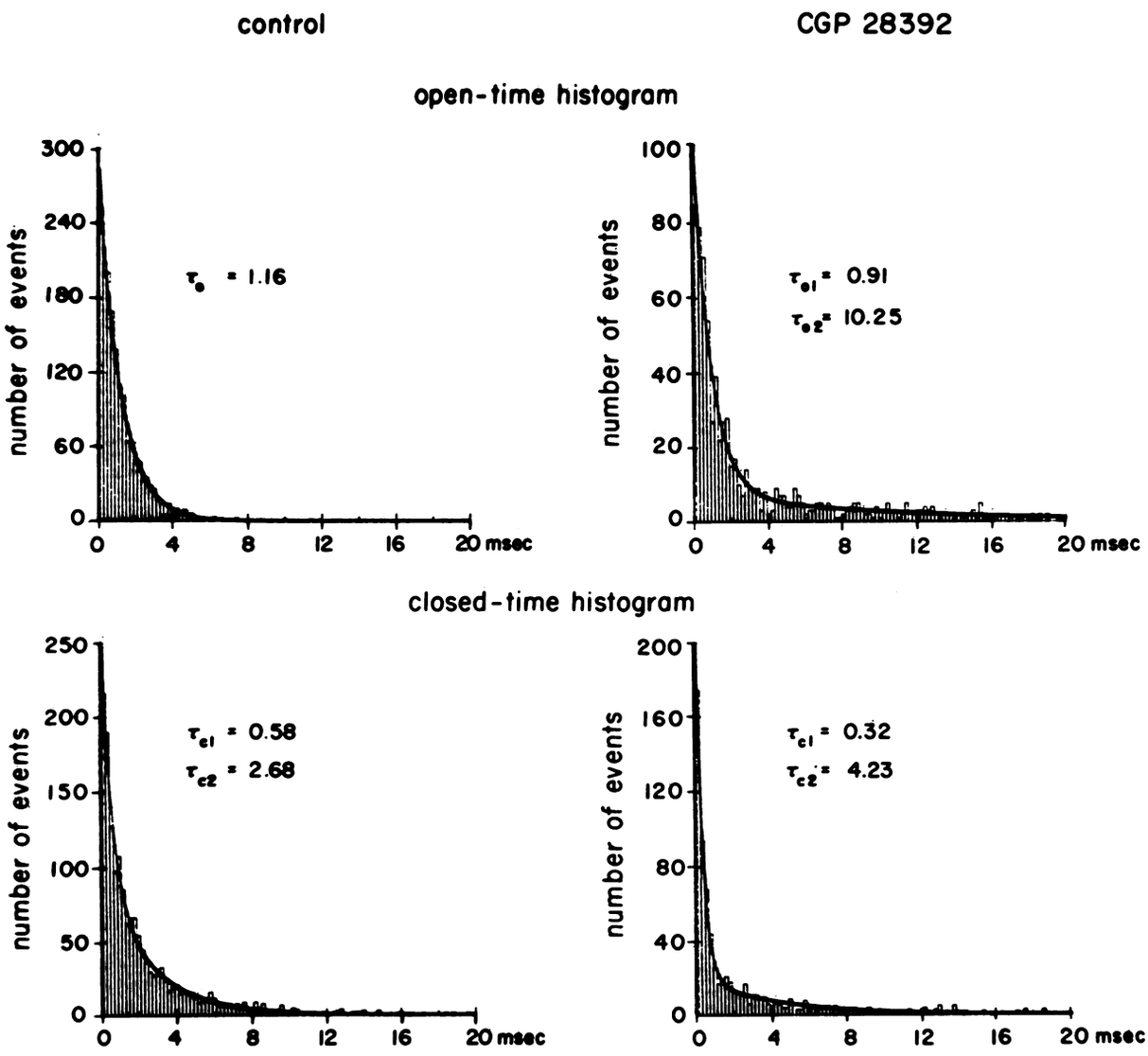

FIG. 3. Open-time histograms (*Upper*) and closed-time histograms (*Lower*) of a Ca channel under control conditions (*Left*) and in the presence of CGP 28392 at ≈1 μM (*Right*). The histograms were fitted with one or two exponentials by means of nonlinear regression analysis. The time constants of the respective exponentials are indicated as insets in the histograms. Note the occurrence of a second exponential in the open-time histogram in the presence of the drug.

with a time constant of 1.16 msec, at least two exponentials were required to fit the open-state histogram of the channel during exposure to the drug. The time constant of the fast exponential was slightly shorter than that in the controls, while the time constant of the second exponential was about an order of magnitude larger. The latter corresponds to the long openings seen in Fig. 1 and reflects the drug-modified state of the channel. The distribution of single-channel closure times was double exponential both in the absence and presence of the drug. The time constant of the fast exponential, which corresponds to the brief closures during bursts of openings, was shorter in the presence of the drug, whereas the number of closures per burst was increased. The intervals between bursts of openings, which are described by the longer time constant of closures, are slightly prolonged in this experiment. However, the prevailing effect of these complicated kinetic changes was the marked prolongation of the open state of the channels. Virtually identical results were obtained with BAY K 8644. With other dihydropyridine derivatives (the Ca channel blockers nimodipine, nitrendipine, and nifedipine), the effect on the long intervals of closure, and particularly an increase in the number of nulls, seemed to be much more pronounced than the effect on the open state of the channel. This led to a reduction of the overall Ca current (unpublished data).

The time constant of the slow component of channel open times (τ_{o_2}) increased with increasing drug concentration, while the ratio of the integrals of the fast and slow components decreased. This led to a concentration-dependent increase in the mean current amplitude (Fig. 4). The averaged current traces (Fig. 4 *Left*) were obtained in one experiment with increasing concentrations of BAY K 8644. The ratios of steady-state Ba^{2+} currents in the presence and absence of the drug are plotted as a dose–response curve (Fig. 4 *Right*). It incorporates the results of four experiments. The dose range for the effects of the drug on single Ca-channel currents was remarkably similar to that for positive effects of the BAY K 8644 in isolated guinea pig hearts (7).

DISCUSSION

The highly lipophilic dihydropyridine derivatives investigated in this study must have access to the Ca channels through the lipid phase of the membrane because no drug was present in the pipette, and even small molecules do not diffuse through the seal area between the rim of the pipette and

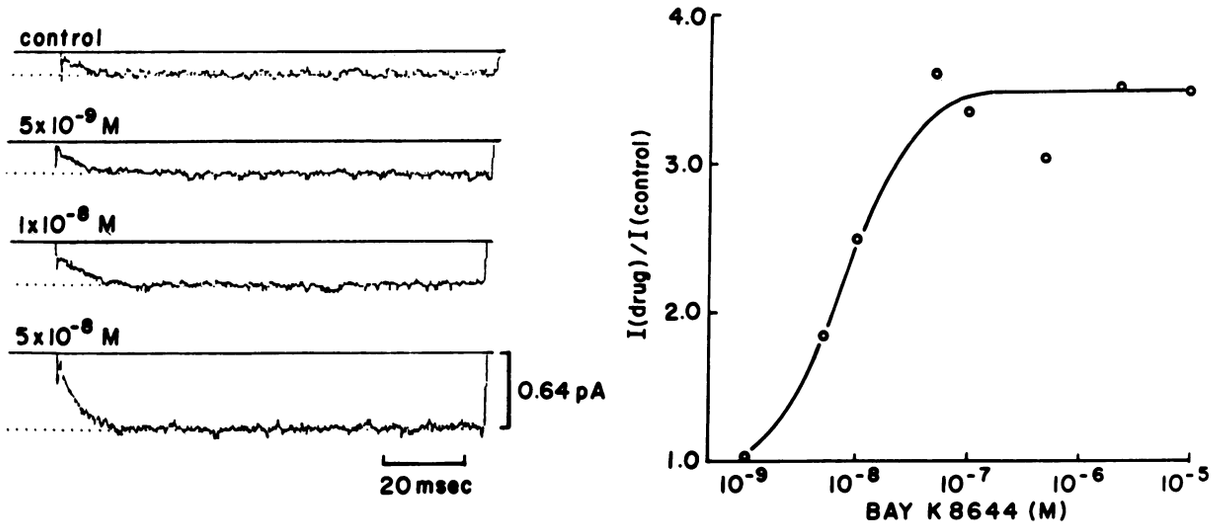

FIG. 4. Dose–response relationships of BAY K 8644 on average Ba^{2+} currents (I). (*Left*) Overall averages of 168–219 single-channel traces, each in the absence (control) and presence of three different BAY K 8644 concentrations measured in a single cell. The pulse potential was 80 mV. (*Right*) Plot of the ratio of average currents in the presence and absence of the drug (ordinate) against drug concentration (abscissa). ○, Mean of four experiments.

membrane surface (8). Because of their very poor water solubility, it is unlikely that the drugs enter into the channel from the cytoplasm. Therefore, this class of drugs may be bound to the channel at or near the channel/lipid interface and, thus, interfere with Ca-channel gating. In contrast to β-adrenoceptor agonists, which modulate Ca-channel activity in cardiac cells indirectly by increasing cyclic AMP in the cell (13–15), we have not found an elevation of cyclic AMP levels by CGP 28392 or BAY K 8644 in our cell system (unpublished data). Furthermore, catecholamines, in addition to slightly increasing mean open times, primarily shorten the intervals between bursts of openings and greatly reduce the number of nulls, thus leading to an enhanced opening probability of Ca channels (11, 14–17). CGP 28392 and BAY K 8644 increase the mean open times to a much greater extent than do catecholamines by promoting the long-lasting open state of the channel, while the number of current sweeps with no openings is not consistently changed.

These results indicate important differences in the mechanisms of Ca-channel modulation by β-adrenoceptor agonists and dihydropyridine derivatives which enhance Ca influx. Investigation of the relationship between the molecular structures of various dihydropyridines and their widely different "agonistic" and "antagonistic" effects on Ca channels may provide new insight not only into molecular mechanisms of action of this important class of drugs but also into structures involved in Ca-channel gating.

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