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Short communication

Ca^{2+} channel activation by CGP 48506, a new positive inotropic benzodiazocine derivative

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Abstract

Effects on L-type Ca^{2+} channels of a new positive inotropic compound, the active (+)-enantiomer of the Ca^{2+} sensitizer 5-methyl-6-phenyl-1,3,5,6,-tetrahydro-3,6,-methano-1,5-benzodiazocine-2,4-dione (CGP 48506), were studied in guinea-pig cardiomyocytes. Whole-cell currents (physiological solutions, 2 mM Ca^{2+}) were enhanced $\approx$ 1.8-fold (10^{-4} M, $n = 7$). Slowing of (de)activation kinetics became apparent under conditions where K^+ currents were fully eliminated and Ca^{2+}-dependent inactivation was minimized ($n = 7$). Single-channel current (70 mM Ba^{2+}) and mean open time were increased $\approx$ 2.5-fold (10^{-4} M, $n = 5$), because the drug specifically enhanced sweeps containing long openings (mode 2). Therefore, CGP 48506 stimulates Ca^{2+} channels in a manner reminiscent of, but not identical to chemically distinct activators like Bay K 8644.

Keywords: CGP 48506; Ca^{2+} channel, L-type; Mode 2 gating; Ca^{2+} channel activator

1. Introduction

Recently, Herold et al. (1995) described a new class of positive inotropic compounds possessing a 1,5-benzodiazocine structure. They reported that only the (+)-enantiomer CGP 48506, between 10^{-5} M and 10^{-4} M, selectively increased calcium sensitivity of skinned cardiac fibres. They concluded that the positive inotropic effects found in the same concentration range were selectively due to this effect at the myofilaments. It is conceptually important to check if this drug indeed represents the first example of a 'pure' calcium sensitizer, devoid of other mechanisms of positive inotropic action. Since Herold et al. (1995) did not rigorously exclude effects on ion channels, we investigated whether this drug affects cardiac L-type calcium channels.

2. Materials and methods

Cell isolations of adult guinea-pig ventricular myocytes for measurements of whole-cell, or single-channel L-type calcium channel currents have been described by Herzig et al. (1995), and Wiechen et al. (1995), respectively. Two series of whole-cell recordings were performed in disposable 1 ml perfusion chambers. In the first series of experiments, bath and pipette solution had 'physiological' composition. Bath solution (mM): NaCl 135, KCl 4, $CaCl_2$ 2, NaH_2PO_4 0.3, $MgCl_2$ 0.5, Hepes 10, dextrose 10 (pH 7.3, 21–23°C); pipette (1.5–2.5 MΩ) solution (mM): K-aspartate 80, KCl 50, KH_2PO_4 10, $MgCl_2$ 0.5, MgATP 3, Hepes 5, EGTA 1 (pH 7.4). Voltage pulses (duration 300 ms, frequency 0.1 Hz) were delivered from a holding potential of −40 mV, usually to +10 mV. Calcium currents were measured as the difference between peak current and the current at the end of the test pulse. In the second series, K^+ was replaced by Cs^+, and intracellular Ca^{2+} was strongly buffered using BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid). In detail, the bath solution contained (mM): CsCl 140, $CaCl_2$ 2, Hepes 10, dextrose 10, pH 7.3, 22–23°C. Pipettes contained (mM): Cs-aspartate 80, CsCl 50, MgATP 4, $MgCl_2$ 1, BAPTA 10, Hepes 10, pH 7.4. Calcium currents were analysed after subtraction of leak and capacitative currents, using a P/N subpulse procedure. Currents were sampled at 6.6 kHz and filtered (−3 dB) at 2 kHz (Axopatch 1D, Axon Instru-

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ments). Drug-containing solution (10^{-4} M, 1% dimethyl sulfoxide (DMSO)) was superfused at 60 ml h^{-1}. Single-channel recordings were done using disposable Petri dishes containing (mM): K-glutamate 120, KCl 25, $MgCl_2$ 2, CaATP 1, EGTA 2, Hepes 10 (pH 7.3, 21–23°C). Pipettes (borosilicate, 7–10 MΩ) were filled with (mM): $BaCl_2$ 70, sucrose 110, Hepes 10 (pH 7.4 with TEA-OH). Pulses (150 ms, 1.6 Hz) were delivered from −100 mV to +20 mV (Axopatch 200A, sampled at 10 kHz, filtered at 2 kHz). Openings were identified by the half-height criterion. Drug was added as a 30 μl bolus (10^{-2} M in DMSO) to the bath, yielding a final concentration of about 10^{-4} M (final bath volume was 3.0 ± 0.15 ml). As examined in previous studies, a DMSO concentration of ≤ 1% (v/v) does not affect calcium channel behaviour under these conditions (Herzig et al., 1993, 1995).

The (+)- and (−)-enantiomers of 5-methyl-6-phenyl-1,3,5,6,-tetrahydro-3,6,-methano-1,5-benzodiazocine-2,4-dione, CGP 48506 (see Fig. 1) and CGP 48508, were obtained from Ciba Geigy (Basle, Switzerland). Other chemicals were of the highest available grade. Data are presented as mean values ± standard errors of n experiments. Significance was checked by two-tailed t-tests (*: $P < 0.05$) using the appropriate (paired or unpaired) format. Single-channel dwell times (open time, closed time, first latency) were analysed by a simple averaging (to yield the indicated mean values). In addition, open time histograms were fitted by exponential functions (first or second order, compared by F test) using the maximum likelihood method (pStat version 6.0, Axon Instruments).

3. Results

CGP 48506 was tested mainly at 10^{-4} M, which has been the maximum concentration used in the inotropy measurements (Herold et al., 1995). This concentration increased whole-cell L-type calcium current amplitude rapidly and reversibly (Fig. 1a). In the $n = 7$ cells tested, the current at +10 mV was enhanced by +77 ± 10%*. At a lower concentration of $2 \cdot 10^{-5}$ M, the currrent was enhanced by only +25 ± 5%* ($n = 3$). Compared with 10^{-4} M CGP 48506, its (−)-enantiomer, CGP 48508 (10^{-4} M), had a weaker but still significant stimulatory effect (+25 ± 6%*, $n = 5$). To investigate the mechanism of channel stimulation, we

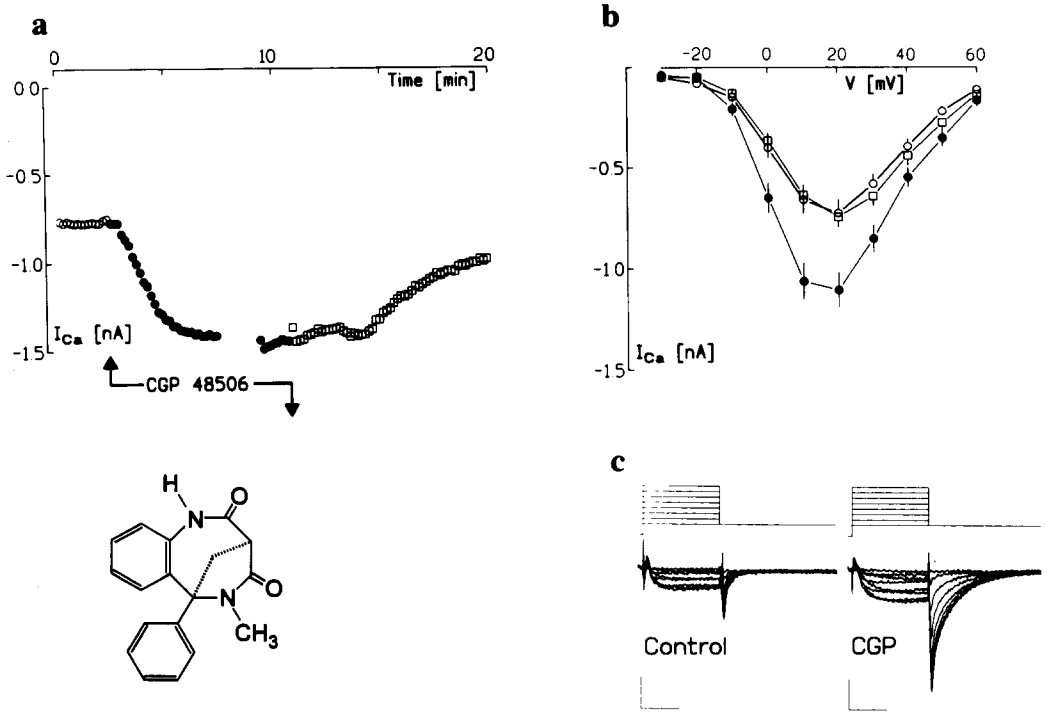

Fig. 1. Structure of CGP 48506 and effects (10^{-4} M) on whole-cell calcium currents in guinea-pig cardiomyocytes. (a) Time course of a single experiment (steps from −40 mV to +10 mV) using 'physiological' solutions. (b) Pooled current-voltage relationships obtained under similar conditions before (○, n = 7) and during (●, $n = 7$) superfusion of the drug, and after washout (□, $n = 5$). (c) Original traces obtained in a cell before (left) and after (right) the addition of 10^{-4} M CGP 48506, with Cs^+- and BAPTA-containing pipette solution (see Materials and methods). The pulse protocol (steps from −40 mV to a test pulse between −20 and +50 mV, then back to −20 mV) is illustrated on top. Scale bars indicate 10 ms and 1 nA.

examined the effects of 10^{-4} M CGP 48506 on the voltage and time dependence of the calcium current. The current-voltage relationship (Fig. 1b) was not shifted along the voltage axis. Likewise, the kinetics of the current waveform appeared unaltered: the peak current was recorded 11.0 ± 0.2 ms after the beginning of the pulse under control conditions, and after 10.5 ± 0.2 ms after drug application (n.s., $n = 7$). However, under the recording conditions used, calcium current kinetics are influenced by Ca^{2+}-dependent inactivation (Lee et al., 1985), and also contaminated by time-dependent K^+ currents. To minimize these problems, intracellular Ca^{2+} was strongly buffered using 10 mM BAPTA in the pipette solution, and K^+ was replaced by Cs^+ in our recording solution. Using the same pulse protocol as above, channel stimulation by 10^{-4} M CGP 48506 again could be observed ($+70 \pm 16\%$, $n = 7$). The current-voltage (IV) relationship was investigated using short test pulses delivered from -40 mV to between -20 mV and $+50$ mV (Fig. 1c), followed by partial repolarization to -20 mV. Again, the peak of the IV curve was not significantly affected (control: 1.4 ± 1.9 mV, drug: 5.7 ± 5.3 mV, n.s., $n = 7$). However, as seen in the original traces (Fig. 1c), a dramatic change in the time course of the current trace became obvious, especially in the tail currents: the rate of deactivation was markedly slowed (τ obtained with monoexponential fits was increased from 3.8 ± 0.3 ms to 10.7 ± 0.9 ms*, $n = 7$). Furthermore, the rate of activation appeared to be retarded. For instance, at a test potential of $+20$ mV, the time from the onset of the test pulse to half-maximum current was significantly delayed (from 3.9 ± 0.1 ms to 4.7 ± 0.2 ms*, $n = 5$ cells). These results indicate that the drug influences the gating behaviour of L-type calcium channels. To examine this effect in more detail, single-channel experiments were done.

Unitary barium currents through L-type channels were recorded in 5 cell-attached (4 single-channel, one double-channel) patches. Peak current amplitude was enhanced by CGP 48506 from 14.2 ± 4.6 to 36.3 ± 11.5 fA*, as exemplified in Fig. 2a. The mechanism of channel stimulation is obvious from the original traces, and from the mean open time diary (Fig. 2b): open times were markedly enhanced, due to the increased abundance of sweeps containing very long openings ('mode 2' sweeps, see Hess et al., 1984; Yue et al., 1990). On average ($n = 5$), mean open time was enhanced by CGP 48506 from 0.44 ± 0.06 ms to 1.11 ± 0.15 ms*. We performed histogram analysis (Fig. 2c,d)

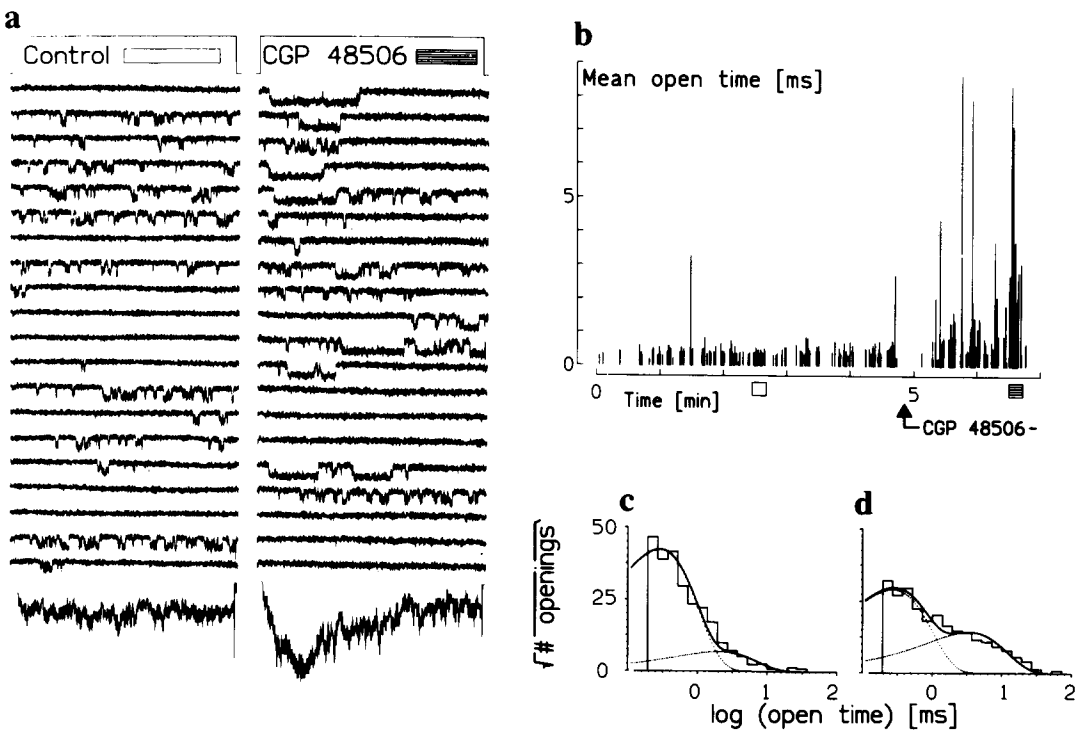

Fig. 2. Effects of CGP 48506 (10^{-4} M) on single-channel behaviour in cell-attached patches. (a) Original traces together with the pulse protocol (top, -100 mV to $+20$ mV) and the pooled averages of this experiment (bottom). Scale bars indicate 20 ms and 25 fA (pooled), or 2 pA (unitary), respectively. (b) Diary of the mean open time per sweep over the entire time course of the same experiment. Boxes indicate positions of the original traces in (a). (c,d) Pooled ($n = 5$ patches) open time histograms before (c) and after drug (d), with second-order exponential fits (solid) and their individual components (dotted).

of all resolved openings pooled from our experiments (7770 events in 2040 control sweeps, and 5023 events in 1910 sweeps after drug). Both data sets required a second-order exponential fit (control: $P < 0.05$, drug: $P < 0.01$), indicating the presence of a short-lived and a long-lived open state. Clearly, the drug caused a redistribution phenomenon: 97.5% of the control openings were short-lived ($\tau_s = 0.28$ ms), leaving a tiny 2.5% component of long openings ($\tau_l = 1.99$ ms). After CGP 48506, this component was amplified to 19.3%, while the time constants remained similar ($\tau_s = 0.27$ ms, $\tau_l = 3.31$ ms).

The increased rate of inactivation seen after CGP 48506 (Fig. 2a) is due to shortening of the period of channel activity (mean burst duration: 69.5 ± 10.0 ms $\rightarrow 42.5 \pm 7.4$ ms*). Other aspects of single-channel gating were not significantly altered by CGP 48506 (mean first latency: 29.0 ± 2.0 ms $\rightarrow 27.0 \pm 3.7$ ms, mean closed time: 4.7 ± 1.0 ms $\rightarrow 2.7 \pm 0.4$ ms, open probability $5.2 \pm 2.0\% \rightarrow 7.7 \pm 1.6\%$). Notably, the channel availability remained unaffected (fraction of sweeps containing channel openings: $24.8 \pm 6.5\% \rightarrow 27.0 \pm 3.7\%$). In summary, stimulation of single calcium channels by CGP 48506 is due exclusively to a change in open times, brought about by the amplification of long-lived openings contained in mode 2 sweeps.

4. Discussion

The inotropic drug CGP 48506 is not a specific calcium sensitizer. In addition to its action on the myofilaments (Herold et al., 1995), it represents a novel type of calcium channel stimulating agent. The mechanism of single-channel action – enhancement of mode 2 gating – resembles that of calcium channel agonists, i.e. dihydropyridines like Bay K 8644 (Hess et al., 1984; Lacerda and Brown, 1989), or FPL 64176 (Kunze and Rampe, 1992). The effects on whole-cell current (de)activation kinetics – although less pronounced – are reminiscent of data obtained with Bay K 8644 (Bechem and Hoffmann, 1993). However, CGP 48506 is structurally unrelated to these compounds. Furthermore, voltage dependence of whole-cell currents is not shifted to the left, in contrast to Bay K 8644 (Sanguinetti et al., 1986; Markwardt and Nilius, 1988), or FPL 64176 (Rampe and Lacerda, 1991). Finally, mode 2 behaviour found with CGP 48506 (Fig. 2) – with open times only moderately enhanced – resembles more closely physiological mode 2 gating induced by phosphorylation (Yue et al., 1990; Zhang et al., 1995) or phosphatase inhibition (Wiechen et al., 1995). In contrast to agents favouring channel phosphorylation (Herzig et al., 1993; Hirano et al., 1994), however, CGP 48506 leaves channel availability unaltered. Furthermore, cAMP content and protein phosphorylation remain unchanged (N. Zimmermann, unpublished). To prove a direct interaction with calcium channels, it is desirable to measure in detail effects of CGP 48506 on binding of all known classes of calcium channel ligands. In addition, direct comparison with Bay K 8644 and FPL 64176, and a detailed study of the voltage and concentration dependence of CGP 48506 action seem worthwhile, given the quantitative differences between single-channel and whole-cell experiments. Furthermore, this compound could help to further understand the functional importance of mode 2. In conclusion, the action of CGP 48506 offers important possibilities to study calcium channel pharmacology and physiology.

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