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Different potencies of dihydropyridine derivatives in blocking T-type but not L-type Ca^{2+} channels in neuroblastoma-glioma hybrid cells

Wolfgang Stengel *, Monika Jainz, Klaus Andreas

Institute of Pharmacology and Toxicology, Faculty of Medicine Carl Gustav Carus, University of Technology, Karl-Marx-Strasse 3, 01109 Dresden, Germany

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Abstract

Evidence has accumulated that classic L-type Ca^{2+} channel blockers with a dihydropyridine structure also inhibit T-type Ca^{2+} channels in certain types of central and peripheral neurons and in smooth muscle cells, albeit with a lower potency. Thus beneficial therapeutic effects of dihydropyridines in cardiovascular and neurological diseases may not only be associated with L-type but also with T-type Ca^{2+} channel blockade. Little is known about the exact order of potency of dihydropyridine derivatives at T-type Ca^{2+} channels. Here we investigate the efficacy and potency of four therapeutically used compounds, i.e. nifedipine, nimodipine, nicardipine, niguldipine, in the neuroblastoma-glioma cell line NG108-15. For comparative purposes the Ca^{2+} channel agonist Bay K 8644 was included. Ca^{2+} channel currents were measured with the whole-cell voltage clamp technique. Subtype Ca^{2+} channel currents were separated by clamp protocol and selective blockers. T-type Ca^{2+} channel currents were inhibited with decreasing potency in the order niguldipine > nicardipine > nimodipine > nifedipine (IC_{50}-values 244 nM, 2.5 μM, 9.8 μM, 39 μM), whereas L-type Ca^{2+} channel currents were blocked with similar potency (IC_{50} for nicardipine 75 nM). Bay K 8644 increased T-type Ca^{2+} channel current at nanomolar concentrations (i.e. 95 $\pm$ 16% increase by 300 nM). T-type Ca^{2+} channel block was completely reversible with exception of the block by niguldipine. Our results indicate a variability of two orders of magnitude in potency of T-type Ca^{2+} channel block by the dihydropyridine derivatives investigated. It is speculated that the relation between the L- and T-type Ca^{2+} channel block may determine the therapeutic profile of a dihydropyridine derivative. © 1998 Elsevier Science B.V.

Keywords: Ca^{2+} channel current; Ca^{2+} channel, T-type; Dihydropyridine derivative; Niguldipine

1. Introduction

T-type Ca^{2+} channels are present in a variety of excitable cells including skeletal (Cognard et al., 1986) and smooth muscle fibers (Loirand et al., 1986), cardiomyocytes (Mitra and Morad, 1986), peripheral (Carbone and Lux, 1984) and central neurons (Akaike, 1991). In neurons of certain brain regions, e.g., in the thalamus, T-type Ca^{2+} channels are responsible for Ca^{2+} spikes (Coulter et al., 1989) and are involved in the transition to phasic bursting behavior (Llinás and Jahnsen, 1982). In combination with Ca^{2+}-activated K^+ currents, membrane potential oscillations are generated which dominate thalamocortical interactions in sleep and under pathophysiological conditions. Thus T-type Ca^{2+} channels are thought to be involved in generalized absence seizures (Steriade and Llinás, 1988). Moreover, T-type Ca^{2+} conductance may also contribute to abnormal cortical synchronous rhythms (Connors and Amitai, 1993). Electrophysiological features of T-type Ca^{2+} currents are variable. In addition, sensitivities to inorganic and organic Ca^{2+} channel blockers differ considerably (Akaike, 1991). Furthermore, the classical L-type Ca^{2+} channel blockers with the dihydropyridine structure also inhibit T-type Ca^{2+} current but with varying potencies. In a study on freshly isolated hypothalamic neurons, T-type Ca^{2+} current was reduced in the order of potency nicardipine > nifedipine > nimodipine with a K_d between 3.5 and 7 μM (Akaike et al., 1989). In hippocampal neurons, nicardipine blocked T-type Ca^{2+} current with an even higher potency than L-type Ca^{2+} current (Takahashi and Akaike, 1990). Since in most of the concentration–response studies on reduction of T-type Ca^{2+} current by dihydropyridines enzymatically dispersed cells were used,

* Corresponding author. Present address: Department of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nürnberg, Krankenhausstrasse 9, 91054 Erlangen, Germany. Tel.: +49-9131-856897; fax: +49-9131-856898.

it was intended to compare T- and L-type Ca^{2+} channel block in cultured cells of the neuroblastoma-glioma hybrid cell line NG108-15. Differently substituted derivatives including nimodipine and nicardipine were studied. The results are discussed on the background of known therapeutical properties of dihydropyridine derivatives, in particular in neuropsychiatric disorders or corresponding animal models. Some of the results have been communicated in preliminary form (Stengel et al., 1996).

2. Material and methods

2.1. Cell culture

NG108-15 mouse neuroblastoma × rat glioma hybrid cells (kindly provided by Professor Wellhöner, Hannover) were grown in a culture medium composed of 90% Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), hypoxanthine–aminopterin–thymidine supplement (HAT), 100 IU/ml penicillin and 100 μg/ml streptomycin. After transferring 3.5×10^4 cells/ml (passage number 20–40) to multiwell-plates (Nunc, Roskilde) containing 10 mm diameter-glass coverslips which had been coated with poly-L-lysine (Sigma, Deisenhofen), cells were cultured for 5 d in a medium which was composed of DMEM with 1% FCS, HAT, the antibiotics and 1 mM dibutyryl-cAMP (Sigma) to induce differentiation. Medium was changed once during the differentiation procedure. Nutrient solutions and sera were obtained from Gibco, Uxbridge.

2.2. Solutions

In the recording chamber (volume 1 ml), cells on the coverslips were superfused (flow rate 1.5 ml/min; about 35°C) with extracellular solution containing (mM): NaCl 145, $BaCl_2$ 10, CsCl 5.5, $MgCl_2$ 1, HEPES 10, glucose 10, tetrodotoxin 0.0001 (pH 7.2). The intracellular (pipette) solution was composed of (mM): $CsCl_2$ 145, $MgCl_2$ 2, EGTA/NaOH 11, EGTA/CaOH 1, HEPES 10, ATP Na_2 2 (pH 7.2).

2.3. Drugs

Drugs were applied using puffing pipettes (4 MΩ resistance, pressure < 1 bar) or a gravity-fed multichannel rapid application system (SPS-8, List Electronics, Darmstadt). Niguldipine which binds strongly to plastic (Boer et al., 1989; Handrock and Herzig, 1996) was only applied by glass-micropipettes. Racemates of the dihydropyridine derivatives nifedipine (Sigma), nimodipine (Tropon-Werke, Köln), nicardipine, niguldipine, Bay K 8644 (RBI, Natick) were prepared as stock solutions (5–30 mM) in dimethylsulfoxide (DMSO). Stock solutions were diluted into the bath solution before experiments. The final concentration of DMSO in the extracellular solution did not exceed 0.1% (v/v) if dihydropyridine effects on L-type Ca^{2+} channel current were studied. DMSO concentrations $\leq 1\%$ (v/v) in the bath solution were applied if dihydropyridine effects on T-type Ca^{2+} channel current were investigated. At 1%, DMSO did not exert a significant effect on T-type Ca^{2+} channel current ($96.4 \pm 3.7\%$ of control, $n = 7$). The effects of dihydropyridine derivatives on voltage-activated Ca^{2+} channel currents were generally assessed after 2–4 min of drug exposure when a steady-state effect had been achieved. On a single cell, one or two dihydropyridine concentrations were tested. Dihydropyridine solutions were protected against light exposure during experiments.

2.4. Recordings and data analysis

Voltage-activated Ca^{2+} channel currents were measured as Ba^{2+} currents in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with an L/M-EPC-7 patch-clamp amplifier (List Electronics). Recording pipettes of 1.5–4 MΩ resistance were fabricated from thin-walled sodium silicate glass capillaries (Vitrex, Paul Block, Strasbourg) by a vertical two-stage electrode puller (L/M-3P-A, List Electronics). Current signals were low pass-filtered (corner frequency 1 kHz), digitized by a 12 bit analog to digital converter (CED 1401, Cambridge Electronic Design, Cambridge) and stored at 3 kHz on a personal computer (486DX33). Current recordings were analyzed using the CED Patch and Voltage Clamp Software 6.0. Capacitive currents in parts were electronically compensated. Series resistance was electronically compensated by about 25%. Voltage-activated Ca^{2+} channel currents were evoked by 200 ms test pulses (frequency 0.1 Hz) in 10 mV increments from a holding potential of -100 mV for T-type Ca^{2+} channel currents and -80 mV for L-type Ca^{2+} channel currents, respectively. With recording pipettes of 4 MΩ resistance, for T-type Ca^{2+} channel currents of 500 pA maximum current amplitude, a voltage clamp with a maximum voltage error of 3 mV was obtained. Criteria for adequate voltage control were absence of abrupt current increase in the rising limbs of the I–V curve and typical kinetics of T-type Ca^{2+} channel currents. Current records were corrected off-line for leakage and residual capacitive currents measured between -80 and -120 mV.

Data points from 3–6 experiments are represented as mean ± S.E.M. Using the software Microcal Origin version 3.5, the logistic function $y = (A_1 - A_2)/\{1 + (x/x_0)^p\} + A_2$ was fitted to the data points of the concentration–response relationships, where A_1 = maximum (% of control), A_2 = minimum (% of control), x_0 = concentration at center of curve, p = slope factor. Unpaired two-tailed Student's test was applied to decide whether drug effects were significant or resulting IC_{50} values did differ significantly (level of significance: $p \leq 0.05$).

3. Results

3.1. Separation of voltage-activated Ca^{2+} channel currents in differentiated NG108-15 neuroblastoma-glioma hybrid cells

Low voltage- and high voltage-activated Ca^{2+} channel currents were distinguished due to their different potential range of activation, potential-dependent inactivation and inactivation kinetics. High voltage-activated current components were pharmacologically separated, since potential range of activation, potential-dependent inactivation and kinetic properties were similar. High voltage-activated currents were characterized by an activation threshold at -20 mV, maximum activation at 0 mV and a half inactivated potential of -24.4 ± 1.9 mV ($n = 7$). For slowly decaying current, time constant of decay was 110 ± 15 ms ($n = 7$) at the test potential 0 mV. Rundown of high voltage-activated Ca^{2+} channel currents of NG108-15 cells was neglected (mean value for 5 min of recording: $3.7 \pm 3.8\%$; $n = 13$). High voltage-activated Ca^{2+} channel currents were separated using 1–10 μM nifedipine to block the

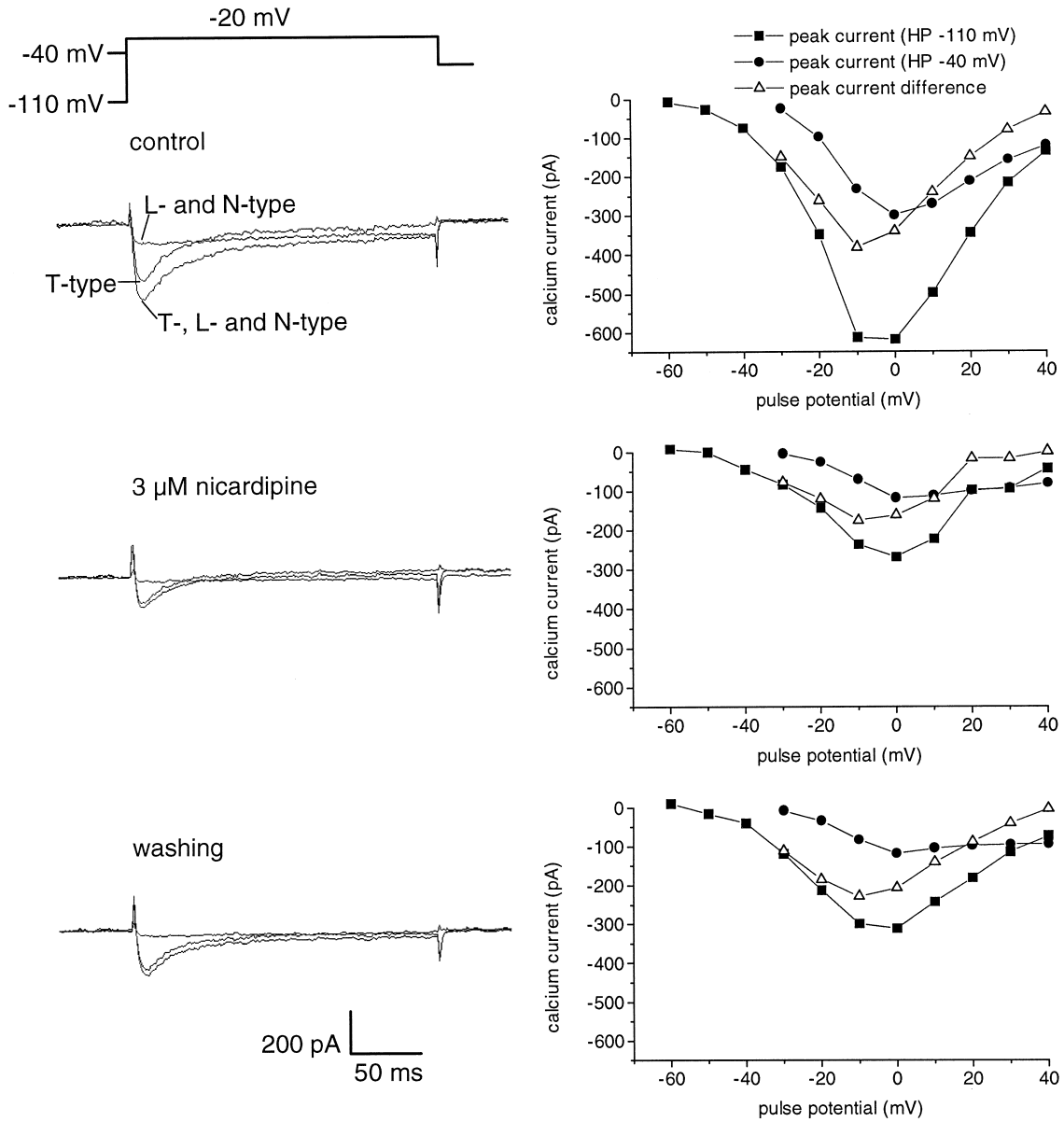

Fig. 1. Effect of nicardipine on low voltage- and on high voltage-activated Ca^{2+} channel currents of a neuroblastoma-glioma hybrid cell. Current records were obtained by 150 ms voltage steps depolarizing in 10 mV increments from 2 holding potentials, -110 and -40 mV, respectively, under control conditions, after 2 min exposure to 3 μM nicardipine and after 4 min of washing. On the left side of the illustration, current traces at a pulse potential of -20 mV, evoked from -110 and -40 mV, and difference of the recordings are represented. On the right, corresponding current to voltage relationships for peak currents are shown. Low voltage-activated current measured as difference peak current was 54% inhibited by 3 μM nicardipine. In this experiment, T- and L-type Ca^{2+} channel block was not reversible after 4 min of washing.

L-type and 10 μM ω-conotoxin GVIA (30 s of exposure) to inhibit the N-type current component. A combination of these drugs reduced high voltage-activated currents to $4.2 \pm 2.0\%$ of control ($n = 6$) indicating that high threshold Ca^{2+} channel current consisted mainly of these two components. Mean L-type Ca^{2+} channel current had a maximum of 438 ± 49 pA ($n = 27$), mean N-type Ca^{2+} channel current of 238 ± 38 pA ($n = 13$). Similar results were obtained if the L-type component was assessed after irreversible inhibition of the N-type component by ω-conotoxin GVIA or if it was determined by the inhibitory effect of 1 μM nifedipine. In contrast to high voltage-activated currents, low voltage-activated Ca^{2+} channel current had an activation threshold at -50 mV and was maximally activated at -20 mV. In order to obtain the low voltage-activated Ca^{2+} channel current in isolation, Ca^{2+} channel currents were evoked from two holding potentials, -110 and -40 mV, and current traces yielded for the same pulse potential were subtracted to eliminate high voltage-activated Ca^{2+} channel currents (Fig. 1). A transient current at a test potential of -20 mV obtained as difference between peak current and sustained current with 150 ms latency was $121.6 \pm 10.0\%$ ($n = 10$) of the peak current resulting from the subtraction procedure. If it is assumed that about 20% of L- and N-type Ca^{2+} channel currents inactivating at -40 mV were not subtracted, T-type Ca^{2+} channel current was completely inactivating during a 200 ms voltage step. In conclusion, transient current at negative potentials was corresponding well to T-type Ca^{2+} channel current. Maximum T-type Ca^{2+} channel current was 191 ± 18 pA ($n = 44$), half inactivated potential was -52.3 ± 5.7 mV ($n = 4$), time constant of decay 14 ± 1 ms ($n = 7$) at the test potential -20 mV.

3.2. Effects of dihydropyridine derivatives on L- and T-type Ca^{2+} channel currents

Drug effects were tested under conditions of complete availability of Ca^{2+} channels (holding potential -80 mV for L-type current, -110 mV for T-type current; pulse frequency 0.1 Hz). First, we examined high affinity Ca^{2+} channel block by the dihydropyridine derivatives nifedipine, nimodipine, nicardipine and niguldipine in differentiated NG108-15 cells. For nimodipine and nicardipine, concentration–response relationships of L-type Ca^{2+} channel current block for concentrations between 10 nM and 1 μM were obtained. Resulting IC_{50} values were 114 ± 27 nM and 75 ± 14 nM, respectively, which did not differ significantly (Fig. 2). In a similar manner, L-type Ca^{2+} channel current reduction by a concentration of 100 nM nifedipine or niguldipine was not different from L-type current block by the same concentration of nicardipine and nimodipine, respectively.

In the second part of the study, the influence of the four dihydropyridine Ca^{2+} channel blockers at nanomolar and micromolar concentrations and of the L-type Ca^{2+} channel agonist Bay K 8644 at two concentrations in the submicromolar range on T-type Ca^{2+} channel current of NG108-15 hybrid cells was assessed. Only niguldipine and Bay K 8644 exerted concentration-dependent effects on T-type Ca^{2+} channel current at submicromolar concentrations (Fig. 3). Whereas Bay K 8644 proved to have agonist properties on T-type Ca^{2+} channel current ($95 \pm 16\%$ increase by 300 nM; $n = 4$), niguldipine reduced T-type current with an IC_{50} value of 244 ± 13 nM ($82 \pm 11\%$ inhibition by the highest concentration used). After appli-

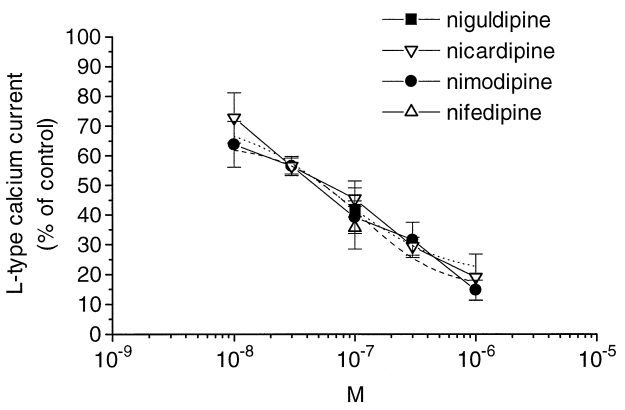

Fig. 2. Concentration-dependent inhibition of L-type Ca^{2+} channel current of neuroblastoma-glioma cells by dihydropyridine derivatives. One or two drug concentrations were tested on a single cell. Each data point was obtained from experiments on 3–5 cells. For separation of subtype Ca^{2+} channel currents, see Section 3. Dashed lines correspond to curves fitted to the data points for nicardipine and nimodipine according to a logistic function. Concentration of half-maximal inhibition was 114 ± 27 nM for nimodipine and 75 ± 14 nM for nicardipine, respectively. Block by 100 nM nifedipine ($64 \pm 7\%$ inhibition) and by 100 nM niguldipine ($58 \pm 7\%$ inhibition) was not significantly different from that exerted by the same concentration of nimodipine and nicardipine, respectively.

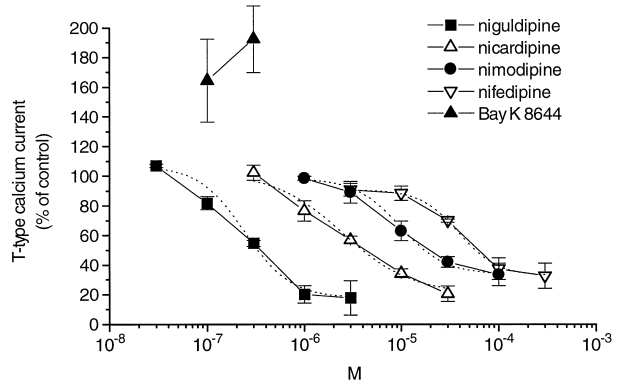

Fig. 3. Concentration–response relationships of the effects of five dihydropyridine derivatives on T-type Ca^{2+} channel current of neuroblastoma-glioma hybrid cells. Whereas Bay K 8644 exerted significant agonist effect at nanomolar concentrations, niguldipine, nicardipine, nimodipine and nifedipine inhibited T-type Ca^{2+} channel current with IC_{50} values of 244 ± 13 nM, 2.5 ± 0.3 μM, 9.8 ± 1.7 μM and 39 ± 5 μM which were obtained from logistic curve fitting indicated by dashed lines. Data points correspond to experiments on 3–6 cells.

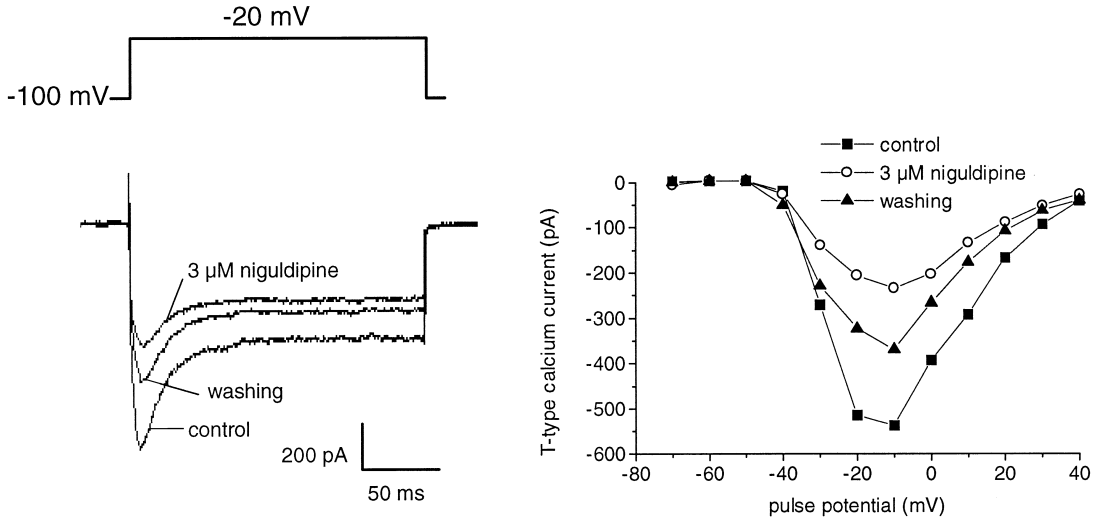

Fig. 4. Effect of niguldipine on Ca^{2+} channel currents of a neuroblastoma-glioma hybrid cell. 3 μM niguldipine was added for 5 min followed by washing up to 10 min. To improve cell survival under clamp conditions, a single clamp step protocol from a hyperpolarized holding potential of -100 mV was used. Original current records at -20 mV and current to voltage relationships for transient current are shown.

cation of 10 μM ω-conotoxin GVIA to inhibit N-type current and in the presence of 1 μM nifedipine to block L-type Ca^{2+} channel current no significant effect of 300 nM Bay K 8644 on T-type current was observed ($92.9 \pm 8.4\%$ of control; $n = 4$). The missing agonist effect may be explained by interactions between the Ca^{2+} channel blocker nifedipine and Bay K 8644. The dihydropyridines nicardipine, nimodipine and nifedipin inhibited T-type current with decreasing potency (half maximum block by 2.5 ± 0.3, 9.8 ± 1.7 and 39 ± 5 μM). Onset of block was rapid, maximum effect was achieved within 2–4 min of exposure to the drugs. In a part of all experiments performed to wash out drugs for 10 min, the effects on T-type current were completely reversible with exception of niguldipine, where recovery generally was only partial (Fig. 4).

4. Discussion

The present results indicate that several dihydropyridine derivatives exhibit widely varying potencies for T-type but similar potencies for L-type Ca^{2+} channel block. In contrast, T-type Ca^{2+} channel block by nicardipine, nimodipine and nifedipine was less variable in a study on freshly isolated hypothalamic neurons (Akaike et al., 1989). It is suggested that structural differences of T-type Ca^{2+} channels account for a lower affinity and potency of nimodipine and nifedipine in the present study. In a similar manner as in NG108-15 cells, potent and reversible effects by nicardipine but not by nifedipine have been reported in acutely isolated dorsal root ganglion neurons (Richard et al., 1991). Niguldipine has been previously shown to block L- and T-type Ca^{2+} channels in cardiac tissue with similar high potencies (Romanin et al., 1992). Here, the drug inhibited L- and T-type Ca^{2+} channel currents of neuronal NG108-15 cells with potencies also in the submicromolar concentration range. This supports the view that niguldipine is a Ca^{2+} channel blocker not discriminating between L- and T-type Ca^{2+} channels. In vascular smooth muscle cells, several dihydropyridines blocked L-type Ca^{2+} current at concentrations (IC_{50} values ≤ 100 nM) similar to those effective in neuroblastoma-glioma hybrid cells whereas markedly higher concentrations of the dihydropyridine derivative CD 832 were required (Friedman et al., 1986; Worley et al., 1986; Kuga et al., 1990; Hirakawa et al., 1994). These findings suggest a uniform potency of L-type Ca^{2+} channel block in neuronal and vascular smooth muscle cells among most of dihydropyridine derivatives.

In our study, potential-dependent and frequency-dependent block of T- and L-type Ca^{2+} channel currents by the dihydropyridine derivatives investigated have not been considered. These effects may be essential for suppression of Ca^{2+} currents under physiological conditions. For nicardipine, an inhibitory effect on T-type Ca^{2+} current of rat hippocampal CA1 neurons which was voltage-, time-, and frequency-dependent has been assessed (Takahashi and Akaike, 1990). Nevertheless, further detailed electrophysiological investigations are required using other derivatives which block potently neuronal T-type Ca^{2+} channel current as it has been shown for niguldipine. Information on T-type Ca^{2+} channel function relies heavily on electrophysiological studies, since up to date no other source of functional information is available: molecular biological methods failed to provide a cloned channel and specific ligands are not as yet known (Zhang et al., 1993; Isom et al., 1994; Tsien et al., 1996). In NG108-15 cells, the potency of T-type Ca^{2+} channel block by the dihydropyridine derivatives was not associated with lipophilicity of the drugs. On the other hand, lipophilic Ca^{2+} channel blockers with a phenylalkylamine or a ben-

zothiazepine structure block T-type Ca^{2+} currents only at high concentrations with IC_{50} values ≥ 30 μM (Akaike et al., 1989; Kuga et al., 1990; Takahashi and Akaike, 1990). This supports specific binding of dihydropyridines at the T-type Ca^{2+} channel. Binding may occur at a conserved region of the α_1-subunit which is functional equivalent to that of the L-type channel and of voltage-dependent Na^+ channels sensitive to higher dihydropyridine concentrations (Yatani et al., 1988). However, an influence of dihydropyridines on regulation of T-type Ca^{2+} channel function cannot been ruled out with the present results. Findings on the level of single channel recording exclude second messenger mediated inhibitory effects of dihydropyridines. In isolated membranes of smooth muscle cells nisoldipine reduced the activity of a high conductance and a low conductance channel correlated with L- and T-type Ca^{2+} channels, respectively (Worley et al., 1986).

Inhibition of T-type Ca^{2+} current in peripheral neurons decreases excitability and may limit sensory afferent input resulting in an analgesic effect. Reduction of T-type Ca^{2+} currents in central neurons may have a broader spectrum of effects. Abnormal T-type Ca^{2+} channel activity in the thalamus has repeatedly been stressed to underly positive symptoms in patients with neurogenic pain, abnormal movements and some types of epilepsy (Jeanmonod et al., 1996). T-type Ca^{2+} current block is considered to be the antiepileptic mechanism of the generalized absence seizure anticonvulsant ethosuximide (Coulter et al., 1990) and possibly of the antiepileptic drug zonisamide effective in partial seizures (Suzuki et al., 1992). The dihydropyridine nicardipine reduced Ca^{2+} spikes of spontaneously epileptic rats at lower concentrations than required to suppress those of normal rats (Momiyama et al., 1995). Moreover, nimodipine suppressed maximal electroshock seizures in rodents (Meyer et al., 1990), whereas nicardipine inhibited pentylenetetrazol-induced seizures (Gasior et al., 1996). Lack of effectiveness of an add-on therapy with nimodipine in epilepsy patients may have been due to insufficient dosages (Meyer et al., 1995). The administration of a dihydropyridine derivative in patients with neurological disorders may be advantageous if cardiovascular effects of the derivative are desirable or are tolerable and may require both drug effects on different voltage-dependent Ca^{2+} channels in an appropriate relationship and convenient pharmacokinetic properties.

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