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Dihydropyridine block of ω-agatoxin IVA- and ω-conotoxin GVIA-sensitive Ca^{2+} channels in rat pituitary melanotropic cells

Huibert D. Mansvelder [a,b], Johannes C. Stoof [b], Karel S. Kits [a,*]

[a] *Graduate School Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, Faculty of Biology, Membrane Physiology Section, De Boelelaan 1087, Amsterdam, 1081 HV, Netherlands*

[b] *Graduate School Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, Department of Neurology, Medical Faculty, Van de Boechorststraat 7, Amsterdam, 1081 BT, Netherlands*

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Abstract

High voltage-activated Ca^{2+} currents in rat melanotropic cells consist of a sustained and an inactivating component. In this study the pharmacological properties of the high voltage-activated Ca^{2+} channels underlying these components are investigated with whole-cell recordings. We report that melanotropes express four pharmacologically distinct high voltage-activated Ca^{2+} channels. Non-inactivating L-type channels account for 35% of the total high voltage-activated channel population. These channels have a very high affinity for the dihydropyridine nimodipine (EC_{50} ~ 3 pM). The cone snail toxin ω-conotoxin GVIA irreversibly blocked an inactivating high voltage-activated component which accounted for 26% of the total whole-cell high voltage-activated Ca^{2+} current. The spider toxin ω-agatoxin IVA reversibly blocked an additional 31% of the total high voltage-activated current. The current blocked by ω-agatoxin IVA was not homogenous and consisted of a sustained component with a high affinity for ω-agatoxin IVA (< 10 nM) and an inactivating current with a low affinity for ω-agatoxin IVA (> 100 nM). Both the ω-agatoxin IVA and ω-conotoxin GVIA-blocked currents were very sensitive to nimodipine and nitrendipine with a half maximal block at 200–500 nM. 10 μM nimodipine blocked 70% of the ω-conotoxin GVIA-sensitive current and 90% of the ω-agatoxin IVA-sensitive current. Thus, ω-conotoxin GVIA- and ω-agatoxin IVA-sensitive high voltage-activated Ca^{2+} channels in melanotropes have an unusual high affinity for dihydropyridines compared to N-, P-, and Q-type channels in other preparations.

Keywords: Ca^{2+} channel; Dihydropyridine; ω-Conotoxin; ω-Agatoxin; Melanotrope; α-MSH (α-melanocyte-stimulating hormone); Pituitary

1. Introduction

Ca^{2+}-dependent intracellular processes are to a large extend controlled by influx of Ca^{2+} through various classes of voltage-sensitive Ca^{2+} channels (Bean, 1989; Hess, 1990; Llinás et al., 1992; Caterall, 1995). In addition to the low voltage-activated T-type channel, until now at least five different types of high voltage-activated channels have been identified, named L, N, P, Q, and R-type (Nowycky et al., 1985; Llinás et al., 1992; Zhang et al., 1993; Sather et al., 1993; Randall and Tsien, 1995). Different types of high voltage-activated channels are coexpressed in various neuronal and endocrine cells (Nowycky et al., 1985; Tsien et al., 1991; Llinás et al., 1992; Artalejo et al., 1992; Williams et al., 1993), but display a differential regulation by neurotransmitters and second messengers (reviewed by Dolphin, 1995) and differences in subcellular distribution (Westenbroek et al., 1992). Thus, a divergence of function of different classes of Ca^{2+} channels may exist within a single cell type.

Initially, much effort was made to separate currents through different high voltage-activated channels on grounds of their biophysical and kinetic properties (Tsien et al., 1988; Carbone and Swandulla, 1989). However, unambiguous identification by kinetic and biophysical properties of channel subtypes underlying the current of a particular cell type was obscured by the presence of, for instance, modal gating properties (Plummer and Hess, 1991; Slesinger and Lansman, 1991), subunit-dependent activation and inactivation rates (Varadi et al., 1991; Zhang et al., 1993; Sather et al., 1993), and overlapping single channel conductances for L, N, and P-type channels (Swandulla et al., 1991; Usowicz et al., 1992; Llinás et al., 1992; Caterall, 1995). As a consequence, the identification

* Corresponding author. Tel.: +31-20-4447096; fax: +31-20-4447123.

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of high voltage-activated Ca^{2+} channels can not be made unambiguously without the aid of pharmacological agents (Hess, 1990; Swandulla et al., 1991).

Although the classification of high voltage-activated Ca^{2+} channels is largely based on pharmacological properties, molecular cloning reveals a channel diversity exceeding the pharmacological classification. The α_1 subunits are encoded for by at least six distinct genes, according to the latest proposed nomenclature called α_{1A} to α_{1E}, and α_{1S} (Birnbaumer et al., 1994), and for most of these several splice variants have been found (Perez-Reyes et al., 1990; Snutch et al., 1991; Williams et al., 1992; Soldatov et al., 1995; Varadi et al., 1995). This implies that identification of channel types on pharmacological grounds is not one-to-one linked to the different α_1-subunit types. For instance, α_1 subunits of L-type channels are encoded for by three genes, i.e. α_{1S}, α_{1C}, and α_{1D} (Williams et al., 1992; for review see Dolphin, 1995). Of these, the α_{1S} and α_{1C} subunits are only sensitive to dihydropyridines, whereas the α_{1D} subunit is also blocked by ω-conotoxin GVIA, ω-agatoxin IVA or the funnel-web spider toxin FTX (Williams et al., 1992; Birnbaumer et al., 1994; Dolphin, 1995). Moreover, the pharmacological sensitivity is not only determined by the α_1 subunit (Perez-Reyes et al., 1989; Sather et al., 1993) but can also be affected by the presence of additional subunits (Caterall, 1995). For example, coexpression of the β subunit increases the dihydropyridine sensitivity of the α_{1C} subunit channel dramatically (Wei et al., 1995) and antisense depletion of β subunits in dorsal root ganglion neurones reduced the sensitivity to the dihydropyridine Bay K 8644 (Berrow et al., 1995). Since β subunits also display a large diversity, i.e. four genes encode for β_1 to β_4 with splice variants of the gene products (Ruth et al., 1989; nomenclature in Birnbaumer et al., 1994; reviewed by Caterall, 1995), a wide range of pharmacological as well as kinetic and biophysical properties can be expected for high voltage-activated Ca^{2+} channels in different cell types.

Melanotropic cells in the intermediate lobe of the pituitary were shown to contain a heterogenous population of Ca^{2+} currents, comprising one rapidly inactivating low voltage-activated T-type current and at least two types of high voltage-activated currents, one of which was inactivating and the other non-inactivating (Cota, 1986; Stanley and Russell, 1988; Williams et al., 1990; Keja et al., 1991, 1992; Keja and Kits, 1994). The sustained component of the high voltage-activated current was shown to be sensitive to dihydropyridines like Bay K 8644 and nifedipine (Keja et al., 1991; Stack and Surprenant, 1991; Williams et al., 1993). Pharmacological approaches by several groups to establish the nature of the inactivating component of the high voltage-activated current resulted in conflicting views. Stack and Surprenant (1991) found it to be reversibly blocked by the N-type channel blocker ω-conotoxin GVIA in addition to a sensitivity to Bay K 8644 and nifedipine. In contrast, Williams et al. (1993), as well as Wang et al. (1992), Beatty et al. (1993) and recently Ciranna et al. (1996), failed to confirm this ω-conotoxin GVIA block and found the inactivating high voltage-activated current to be blocked by the P-type channel blocking polyamine FTX instead (Williams et al., 1993). However, they did not look into any overlap with dihydropyridine sensitivity.

In this study we address the question of the pharmacological sensitivity of the heterogeneous high voltage-activated Ca^{2+} channel population in melanotropes in more detail. We report here that most of the sustained component of the high voltage-activated current is blocked with a very high affinity by dihydropyridines, reminiscent of the high affinity block of L-type channels in aorta cell-lines (McCarthy and Cohen, 1989). In addition, a small part of the sustained current appears to be blocked by low concentrations of ω-agatoxin IVA. The inactivating high voltage-activated Ca^{2+} current in cultured melanotropes is carried by ω-conotoxin GVIA-sensitive and ω-agatoxin IVA-sensitive channels, which, in addition, display an unusual sensitivity to the dihydropyridines nimodipine and nitrendipine.

2. Materials and methods

2.1. Animals and culture

Rat (male Wistars, 1.5–2 months of age, 200–250 g, Harlan CPB, Netherlands) pituitary melanotropic cells were isolated as described previously (Keja et al., 1991). The cells were cultured on poly-L-lysine coated coverslips at a density of 0.5 intermediate lobe per coverslip. The culture medium was changed every third day and consisted of Biorich I (Flow), Hepes 10 mM, Ultroser G 5% (Gibco), penicillin G 200 U/ml (Sigma), streptomycin 50 μg/ml (Sigma), and cytosine arabinosine 1 μM (Sigma) adjusted to pH 7.2 with NaOH. Cells were maintained up to 10 days in a 37°C incubator with a humidified atmosphere comprising 5% CO_2 in air.

2.2. Electrophysiological recordings

Coverslips bearing melanotropic cells were transferred to the recording chamber containing 300 μl external solution. The external solution contained (in mM): tetraethylammonium chloride 130, glucose 10, $BaCl_2$ 13, Hepes 10, 4-aminopyridine 1, pH 7.4, adjusted with tetraethylammonium hydroxide. Electrodes (impedance after fire polishing: 3–6 MΩ) were pulled on a Flaming/Brown P-87 (Sutter Instruments, CO) horizontal microelectrode puller from Clark GC-150 borosilicate glass (Clark Electromedical Instruments, England). The pipette solution contained (in mM): $CsCl_2$ 135, $MgCl_2$ 2, $CaCl_2$ 1, Hepes 10, EGTA 11, MgATP 2, TrisGTP 0.1, pH 7.4, adjusted with CsOH. Whole cell voltage clamp recordings were performed at room temperature and were obtained from

spherical cells, 10–15 μm in diameter, with a bright halo, previously shown to contain α-melanocyte-stimulating hormone (α-MSH) and other pro-opiomelanocortin products by immunostaining and single cell MALDI-mass spectrometry (Keja et al., 1991; C.R. Jiménez and H.D. Mansvelder, unpublished). Current recordings were filtered at 1 kHz, using the 4-pole low-pass Bessel filter of the Axopatch 200A amplifier (Axon Instruments, USA), before digitizing with a Digidata 1200 (Axon Instruments) at a sampling frequency of 4 kHz. Series resistance was typically lower than 8 MΩ and was not compensated in order to reduce noise. Voltage-command errors due to series resistance were estimated to be lower than 2,4 mV (300 pA and 8 MΩ). The whole-cell capacitance was generally 3–5 pF and was compensated. Recordings were made at a holding potential of −80 mV and effects of pharmaca and toxins were tested at a potential of +10 mV. The effects of the pharmaca and toxins were measured when a steady state level of block was reached. Experiments were performed and analyzed using Pclamp v6.02 software (Axon Instruments, USA). The Systat software (USA) was used for statistical analysis and the fitting of the data was done using the NLREG v3.3 software of Ph.H. Sherrod (Nashville, TN, USA). Error bars presented signify the standard errors of the mean (S.E.M.). Mean values of the data are given ±S.E.M. unless stated otherwise.

2.3. Drugs and application

All pharmaca were applied either by a gravity-driven Y-tube system or by pressure ejection. In addition, the recording chamber was continuously perfused at a rate of ~ 1.5 ml/min, driven by air pressure, and the bath volume was kept constant by continuous suction. Nitrendipine (1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid ethyl methyl ester) and nimodipine (1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinecarboxylic acid 2-methoxyethyl 1-metylethyl ester) were purchased from RBI (USA). Calciseptin was obtained from Latoxan (France). ω-Conotoxin GVIA and ω-agatoxin IVA were a generous gift from Dr B.M. Olivera, University of Utah (Salt Lake City, UT, USA).

3. Results

Whole cell voltage clamp recordings from 139 cultured melanotropes revealed a heterogenous population of Ca^{2+} currents, comprising a fast inactivating low voltage-activated T-type current, and multiple high voltage-activated currents differing in inactivation kinetics, as shown previously (Cota, 1986; Stanley and Russell, 1988; Williams et al., 1990; Stack and Surprenant, 1991; Keja et al., 1991; Keja and Kits, 1994). During the first 5–10 min

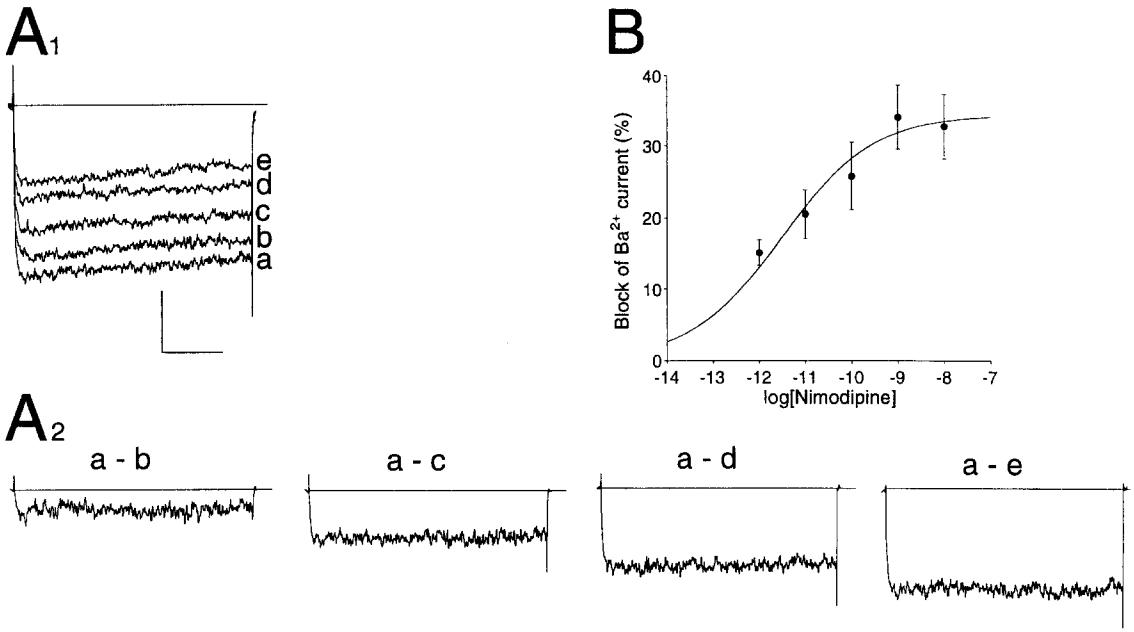

Fig. 1. High affinity block of a sustained high voltage-activated current by nimodipine. (A_1) Example of an experiment with increasing concentrations of nimodipine tested on a single cell. Whole-cell Ba^{2+} currents were elicited by a 200 ms testpulse from −80 mV holding potential to +10 mV test potential. Trace a: control current; trace b–e: current in the presence of 1, 10, 100, and 1000 pM nimodipine, respectively. (A_2) Subtractions of control trace a and traces (b–e) in the presence of increasing concentrations of nimodipine, showing the current blocked by 1–1000 pM nimodipine. Scale bars in A_1: horizontal 50 ms; vertical 100 pA. (B) Dose-effect curve of high affinity block by nimodipine ($n = 5$ to 15 for each data point). The data were fitted by the equation: $Y = \mathrm{Inh}_{max} \cdot \frac{C}{(C + \mathrm{EC}_{50})}$; where Y is the percentual inhibition of the whole-cell Ba^{2+} current, Inh_{max} (= 34.5%) the inhibition at the plateau level, C is the nimodipine concentration, and EC_{50} (= 3 pM) the concentration that produces half-maximal block.

after establishment of the whole cell configuration, the Ba^{2+} current increased to a steady state level, which held for at least 20 min and during which period the experiments were performed. When cells were stepped from a holding potential of −80 mV to +10 mV during a test potential of 200 ms the maximal high voltage-activated current was activated and had an average amplitude of −186 ± 93.6 pA (mean ± S.D.).

3.1. High affinity block by nimodipine

The dihydropyridine nimodipine has been reported to block selectively high voltage-activated L-type channels with high affinity in various neuronal and non-neuronal cell types (McCarthy and Cohen, 1989; reviews by Bean, 1989; Hess, 1990). Nimodipine appeared to block the whole cell high voltage-activated Ba^{2+} current in melanotropes at very low concentrations. Fig. $1A_1$ shows the concentration-dependent block of the whole cell Ba^{2+} current. Subtractive determination of the waveforms of the current blocked by different concentrations of nimodipine showed that nimodipine blocked a non-inactivating current (Fig. $1A_2$). The concentration dependence of the block by nimodipine was analyzed by fitting a dose-response curve, assuming a single binding site (Fig. 1B). The EC_{50} of the best fit was ~ 3 pM, and maximal block was obtained at 1 nM (34.1 ± 4.5%; $n = 10$). Thus, one third of the whole-cell Ba^{2+} current is carried by L-type channels. This is in accordance with the findings of Williams et al. (1990), Keja et al. (1991), and Stack and Surprenant (1991). In addition, the peptidergic L-type channel blocker calciseptine blocked the high voltage-activated current by 30.6 ± 3.4% ($n = 3$; Fig. 2A,B). This was not significantly different from the average block by 1 nM nimodipine (unpaired Student's t-test, $P = 0.554$). Subtraction of control traces and traces in the presence of 5 μM calciseptine showed that the blocked current again was non-inactivating (Fig. $2A_2$), confirming that one third of the total Ba^{2+} current is a sustained current carried by L-type channels.

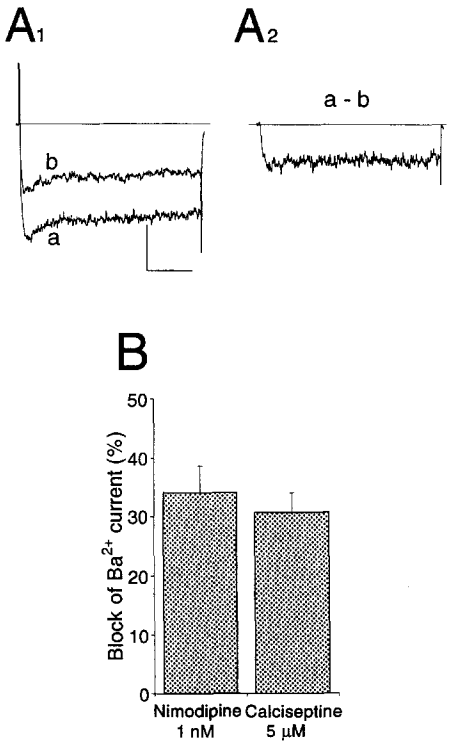

Fig. 2. Block of a sustained high voltage-activated Ca^{2+} current by the peptidergic L-type channel blocker calciseptine. (A_1) Effect of 5 μM calciseptine. Trace a is control, trace b in the presence of calciseptine. (A_2) Subtraction of trace a and b of A_1 shows that 5 μM calciseptine blocked a sustained current. Scale bars: horizontal 50 ms; vertical 50 pA. (B) Average block of whole-cell Ba^{2+} current by 1 nM nimodipine ($n = 10$) and 5 μM calciseptine ($n = 3$). The averages are not significantly different (unpaired Student's t-test $P = 0.554$).

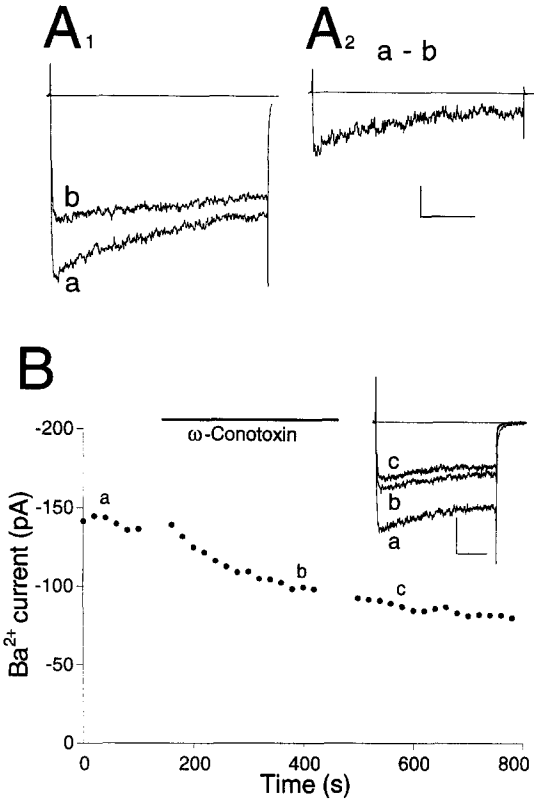

Fig. 3. Effects of ω-conotoxin GVIA on the whole-cell Ba^{2+} current. (A) Left: example of an experiment with 1 μM ω-Conotoxin GVIA. Trace a is the control current, trace b the current in the presence of 1 μM ω-Conotoxin GVIA. Right: the ω-conotoxin GVIA sensitive current obtained by subtraction. This current inactivates substantially during a 200 ms testpulse. (B) Time course of the effect of 1 μM ω-conotoxin GVIA. Application of 1 μM ω-conotoxin GVIA is indicated by the horizontal bar. The inset shows current traces at the points marked a (control), b (in the presence of 1 μM ω-conotoxin GVIA), and c (wash out). Scale bars in (A) and the inset of (B): horizontal 50 ms; vertical 50 pA.

3.2. Block by ω-conotoxin GVIA

ω-Conotoxin-GVIA (1 μM), a cone snail toxin that selectively blocks N-type channels in other neurons (Aosaki

and Kasai, 1989; Plummer et al., 1989), blocked the high voltage-activated current by 25.5 ± 3.6% ($n = 13$; Fig. 3A$_1$). The block at 10 μM ω-conotoxin GVIA was 22.9 ± 2.5% (not significantly different from the block by 1 μM; $n = 3$), indicating that 1 μM ω-conotoxin GVIA yields a saturating effect. Subtractive determination of the ω-conotoxin GVIA-sensitive current revealed that it inactivates during a 200 ms testpulse (Fig. 3A$_2$). This is in accordance with the findings of Stack and Surprenant (1991) who reported a 25 ± 6% block of the transient component of the high voltage-activated current in melanotropes. However, in contrast to their findings, the block of 1 μM ω-conotoxin GVIA in our melanotropic cell culture did not reverse in 6–10 min wash (Fig. 3B). These results demonstrate the presence of an inactivating current with pharmacological properties reminiscent of N-type channels in other tissues (McCleskey et al., 1987; Aosaki and Kasai, 1989; Plummer et al., 1989; for reviews see Hess, 1990; Swandulla et al., 1991).

3.3. Block by ω-agatoxin IVA

The funnel web spider toxin ω-agatoxin IVA is known to block a distinct class of Ca^{2+} channels (Mintz and Bean, 1993) which accounts for the major component of the high voltage-activated Ca^{2+} current in cerebellar Purkinje neurons and is therefore called P-type (for review see Llinás et al., 1992). In melanotropic cells ω-agatoxin IVA blocked the high voltage-activated current by 30.9 ± 6.0% (100 nM; $n = 5$;) with a half maximal block at 5-10 nM (Fig. 4A). The time course of block was similar to the time course of ω-agatoxin IVA block in cerebellar granule cells (Randall and Tsien, 1995), with a maximal inhibition by 100 nM within 3–4 min (Fig. 4B). However, in contrast to the block in cerebellar granule cells (Randall and Tsien, 1995) and in cerebellar Purkinje cells (Mintz and Bean, 1993), the block of ω-agatoxin IVA in melanotropes reversed upon wash out of the toxin in ~ 5 min (Fig. 4B).

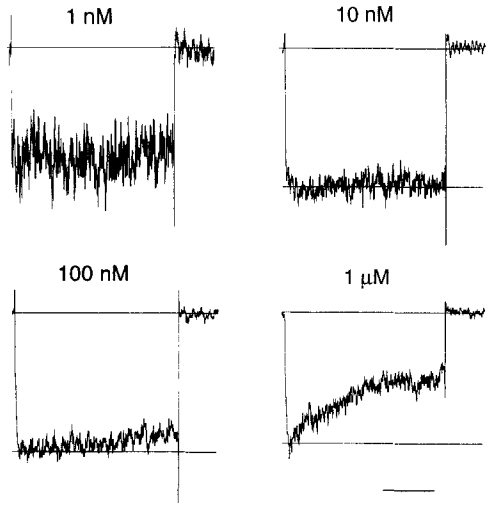

Fig. 5. Dose dependence of the waveforms of the ω-agatoxin IVA-blocked currents. Normalized averaged waveforms of the ω-agatoxin IVA-sensitive currents at different concentrations of ω-agatoxin IVA, as indicated above the traces, are calculated from the same cells as in Fig. 4A. Scale bar: 50 ms.

The waveform of the ω-agatoxin IVA-sensitive current at different concentrations of ω-agatoxin IVA was determined by subtracting control and test traces (Fig. 5). The waveform of the ω-agatoxin IVA-sensitive current in melanotropic cells was dependent on the concentration of the toxin. At low concentrations, i.e. 1 nM and 10 nM, the blocked current did not inactivate during a test potential of 200 ms at + 10 mV. At higher concentrations, 100 nM and 1 μM, the current did inactivate during the test pulse, with a little decay at 100 nM and a much larger decay at 1 μM (Fig. 5). These results are similar to those in cerebellar granule cells, where ω-agatoxin IVA blocked a sustained P-type current at low concentrations (~ 1 nM) and, additionally, an inactivating Q-type current with low affinity (~ 100 nM) (Randall and Tsien, 1995). Thus, our data

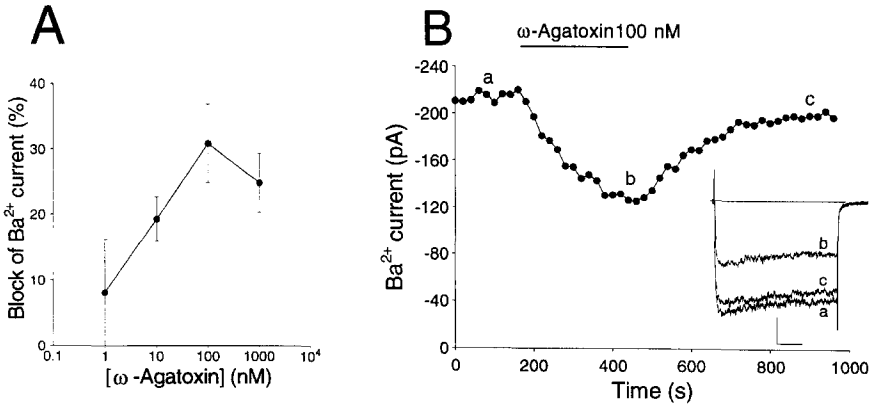

Fig. 4. Effects of ω-agatoxin IVA on the whole-cell Ba^{2+} current. (A) Dose dependence of the block by ω-agatoxin IVA curve. Each data point represents the average of 3–9 cells and per cell one concentration ω-agatoxin IVA was tested, giving rise to a considerable variation in the amount of whole-cell current block by ω-agatoxin IVA. Therefore, no dose-response curve was fitted to the data. (B) Time course of the effect of 100 nM ω-agatoxin IVA. The inset shows current traces taken at the points marked a (control), b (in the presence of 100 nM ω-agatoxin IVA), and c (wash out). Scale bars of the inset: horizontal 50 ms and vertical 50 pA.

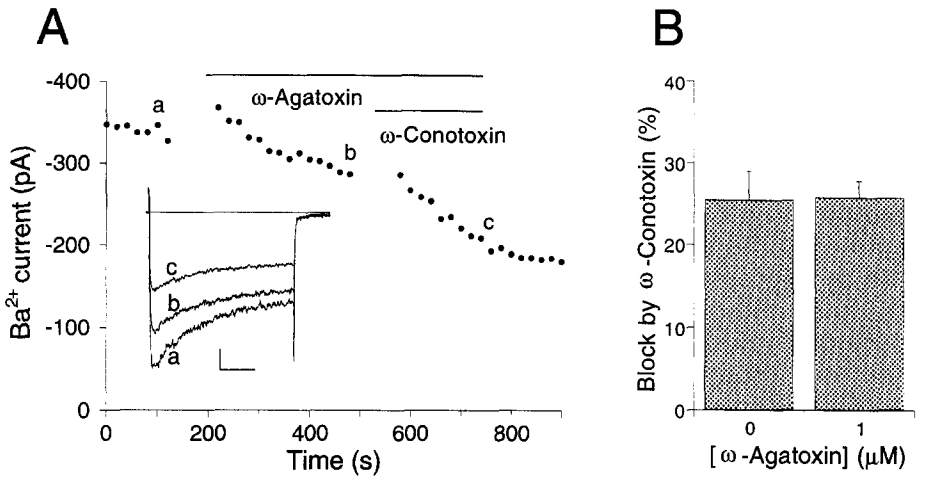

Fig. 6. Effects of ω-agatoxin IVA and ω-conotoxin GVIA are additive. (A) Plot of the Ba^{2+} current amplitude vs. time under control conditions and with subsequent application of 1 μM ω-agatoxin IVA alone and ω-agatoxin IVA together with 1 μM ω-conotoxin GVIA (as indicated). The block by ω-conotoxin GVIA clearly adds to the block by ω-agatoxin IVA. The inset shows whole current traces at the points marked a (control), b (in the presence of 1 μM ω-agatoxin IVA), and c (in the presence of 1 μM ω-agatoxin IVA and 1 μM ω-conotoxin GVIA). Scale bars of inset: horizontal 50 ms; vertical 50 pA. (B) Average block by 1 μM ω-conotoxin GVIA in the absence ($n = 7$) and in the presence ($n = 5$) of 1 μM ω-agatoxin IVA. There was no significant difference between the averages (unpaired Student's t-test, $P > 0.5$).

suggests a heterogeneity of the ω-agatoxin IVA-sensitive high voltage-activated Ca^{2+} current in melanotropes comprising a non-inactivating, P-type-like, current with a high sensitivity to ω-agatoxin IVA (concentrations < 10 nM), and a rapidly inactivating, Q-type-like, current with a low toxin sensitivity (> 100 nM).

3.4. ω-Conotoxin GVIA and ω-agatoxin IVA block distinct components of the high voltage-activated current

Subtractive determination of the waveforms of the currents blocked by ω-conotoxin GVIA and 1 μM ω-agatoxin IVA revealed that both toxins block an inactivating high voltage-activated current (Fig. $3A_2$ and Fig. 5). To determine whether these toxins block the same inactivating component of the high voltage-activated current in melanotropes, the toxins were applied in combination. Fig. 6 shows that the presence of 1 μM ω-agatoxin IVA did not affect the amount of block of the high voltage-activated current by 1 μM ω-conotoxin GVIA. In the absence of ω-agatoxin IVA, ω-conotoxin GVIA blocked 25.5 ± 3.6% ($n = 7$) of the current, while in the presence of 1 μM ω-agatoxin IVA, ω-conotoxin GVIA blocked 25.8 ± 2.0% ($n = 5$) of the current (not significantly different; $P > 0.5$, unpaired Student's t-test; Fig. 6B). Thus, the block of ω-conotoxin GVIA and ω-agatoxin IVA was fully additive, suggesting that each of the toxins blocks a distinct component of the inactivating Ca^{2+} current.

In addition to a difference in pharmalogical sensitivity, the high voltage-activated currents blocked by ω-agatoxin IVA and ω-conotoxin GVIA differed with respect to their activation rate. Fig. 7A shows the first 20 ms of the averaged currents blocked by ω-agatoxin IVA and by ω-conotoxin GVIA (both $n = 5$); for comparison also the current blocked by 1 nM nimodipine is shown ($n = 10$). The ω-conotoxin GVIA-sensitive current activated slower

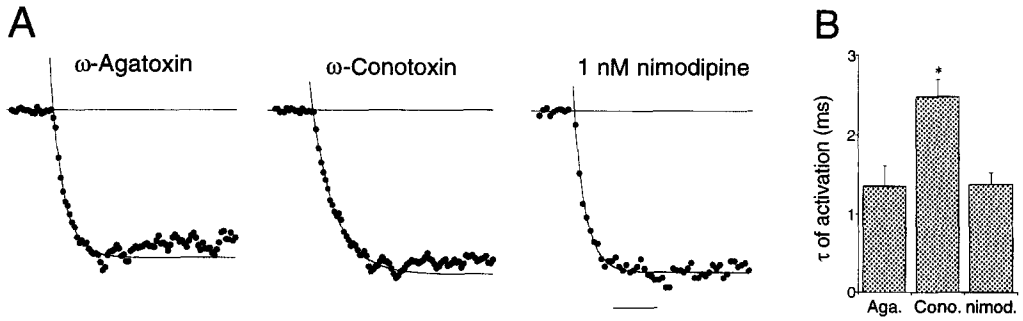

Fig. 7. Activation kinetics of the ω-agatoxin IVA, ω-conotoxin GVIA, and nimodipine sensitive currents. (A) Activation kinetics of averaged and normalized whole-cell Ba^{2+} current components characterized by their toxin sensitivity as indicated. The rising phases were fitted by mono-exponential activation curves. Scale bar 5 ms. (B) The average τ_{act}'s as determined from single experiments. The asterisks denote a significant difference between the τ_{act} of the ω-conotoxin GVIA-blocked current versus those of the ω-agatoxin IVA- and nimodipine-blocked currents ($P < 0.01$; unpaired Student's t-test).

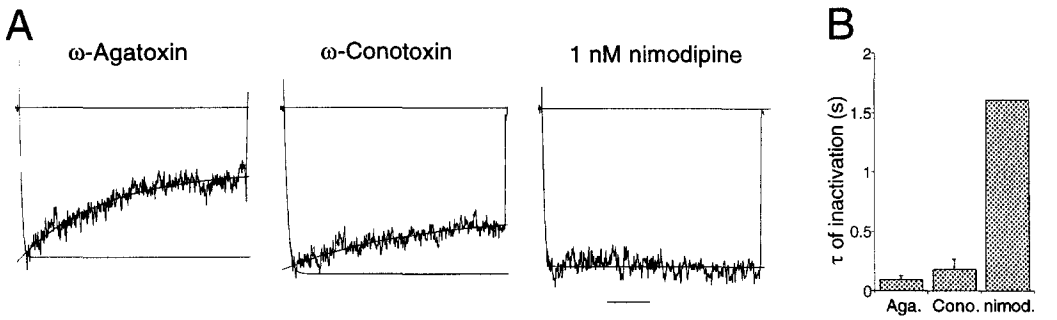

Fig. 8. Inactivation kinetics of the ω-agatoxin IVA, ω-conotoxin GVIA, and nimodipine sensitive currents. (A) Same traces as in Fig. 7A but on a larger time scale (scale bar 50 ms). The decaying phases of the ω-agatoxin IVA and ω-conotoxin GVIA-blocked currents were fitted with a mono-exponential decaying curve. The fits of the activating phase of 7A are also shown. (B) The average τ_{inact} as determined from single experiments (no significant difference for the ω-agatoxin IVA and ω-conotoxin GVIA blocked currents). The value of the τ_{inact} of the L-type current (1600 ms) was taken from Keja et al. (1991).

than the currents blocked by ω-agatoxin IVA and 1 nM nimodipine. The activation phase of each single experiment was fitted by a mono-exponential function and the resulting time constant (τ_{act}) was averaged for each of the pharmaca. Fig. 7B shows that the τ_{act} of the ω-agatoxin IVA-sensitive current, 1.35 ± 0.26, and the τ_{act} of the 1 nM nimodipine-sensitive current, 1.38 ± 0.15 ms, were almost twice as low as that of the ω-conotoxin GVIA-sensitive current, which was 2.48 ± 0.22 ms (significantly different from both the ω-agatoxin IVA and nimodipine-sensitive currents; $P < 0.01$, unpaired Student's t-test). These data strongly suggest that ω-conotoxin GVIA blocks a component of the inactivating high voltage-activated current which is distinct from the inactivating ω-agatoxin IVA-sensitive current.

In contrast to the time constants of activation, the time constants of inactivation (τ_{inact}) of both inactivating components of the high voltage-activated current were not significantly different (Fig. 8), which implies that the inactivating components cannot be separated by means of their inactivation rate. Toxin-blocked currents resulting from single experiments were fitted with a mono-exponentially decaying function and the time constants were averaged for each of the toxins. The average τ_{inact} of the ω-agatoxin IVA-blocked current was 101.7 ± 28.4 ms and that of the ω-conotoxin GVIA-blocked current $180.9 \pm$

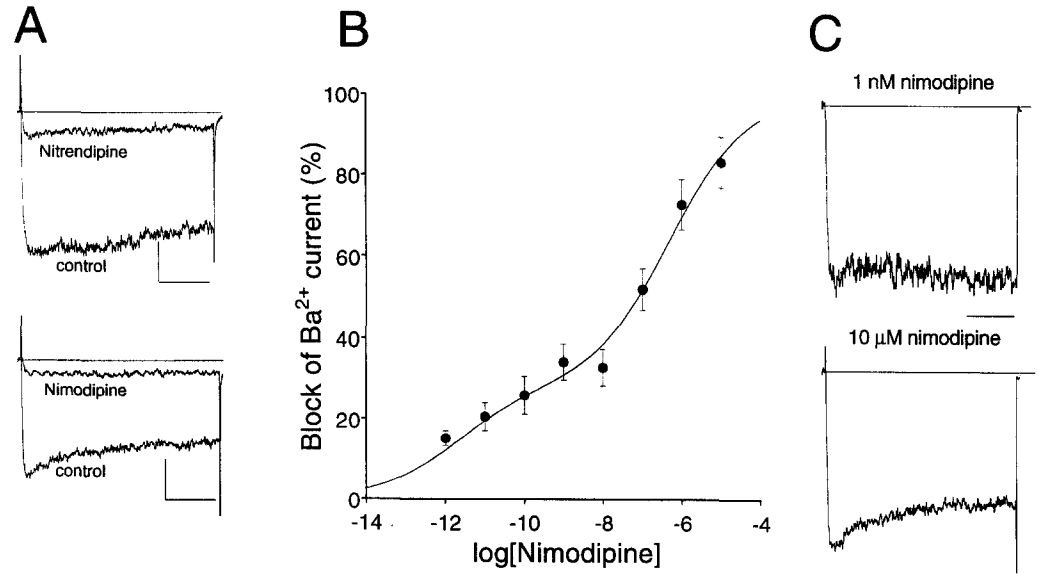

Fig. 9. Effects of high concentrations of nimodipine and nitrendipine. (A) Examples of effects of 10 μM nitrendipine and nimodipine on the whole-cell Ba^{2+} current. Scale bars: horizontal 50 ms, vertical 50 pA. (B) Dose-effect curve of the block by nimodipine in concentrations from 1 pM to 10 μM. The data were best be fitted by a two-site binding curve of the form $Y = \mathrm{Inh}_{max}(1) \cdot \frac{C}{(C + \mathrm{EC}_{50}(1))} + \mathrm{Inh}_{max}(2) \cdot \frac{C}{(C + \mathrm{EC}_{50}(2))}$, where $\mathrm{Inh}_{max}(1) = 28\%$, $\mathrm{Inh}_{max}(2) = 72\%$, $\mathrm{EC}_{50}(1) = 3$ pM, and $\mathrm{EC}_{50}(2) = 500$ nM. Data points are averages of 5 to 17 experiments. The data of 1 pM to 10 nM nimodipine are taken from Fig. 1B. (C) Waveforms of the current blocked by 1 nM nimodipine (n = 10) and 10 μM ($n = 10$), as indicated. The nimodipine sensitive currents were determined in each experiment by subtraction, these traces were normalized and the normalized traces were then averaged. Scale bar is 50 ms.

89.5 ms (Fig. 8B). The slowly inactivating L-type current was previously shown to inactivate with a time constant of 1600 ms (Keja et al., 1992).

3.5. Block of the toxin-sensitive, inactivating high voltage-activated currents by high concentrations of dihydropyridines

Stack and Surprenant (1991) reported that the transient as well as the sustained high voltage-activated Ca^{2+} current in melanotropic cells was sensitive to high concentrations (> 1 μM) of dihydropyridines. In our melanotropic cell culture, high concentrations (10 μM) of the dihydropyridines nimodipine and nitrendipine blocked 83.2 ± 6.3% ($n = 10$) and 84.9 ± 2.7% ($n = 6$), respectively, of the high voltage-activated current (Fig. 9A), suggesting dihydropyridine block of the toxin-sensitive inactivating high voltage-activated currents. The dose-response curve of nimodipine in Fig. 9B shows the appearance of an extra component when concentrations above 10 nM are used. The data were best fitted by a two binding-site curve with a half maximal block, $EC_{50}(1)$, of ~ 3 pM for the high affinity site and an $EC_{50}(2)$ of ~ 500 nM for the low affinity site. Subtractive determination of the high voltage-activated component blocked by 10 μM nimodipine showed that nimodipine indeed blocks an inactivating high voltage-activated component (Fig. 9C).

To determine whether the ω-conotoxin GVIA-sensitive or the ω-agatoxin IVA-sensitive, or both currents were blocked by nimodipine, the amount of block by each of the toxins in the presence of increasing concentrations of nimodipine was determined. The block of the high voltage-activated current by 1 μM ω-conotoxin GVIA was substantially affected by the presence of nimodipine (Fig. 10A,B). The amount of block of the high voltage-activated current by ω-conotoxin GVIA is presented as percentage of the initial control, where neither nimodipine nor ω-conotoxin GVIA was present. Fig. 10B shows that nimodipine

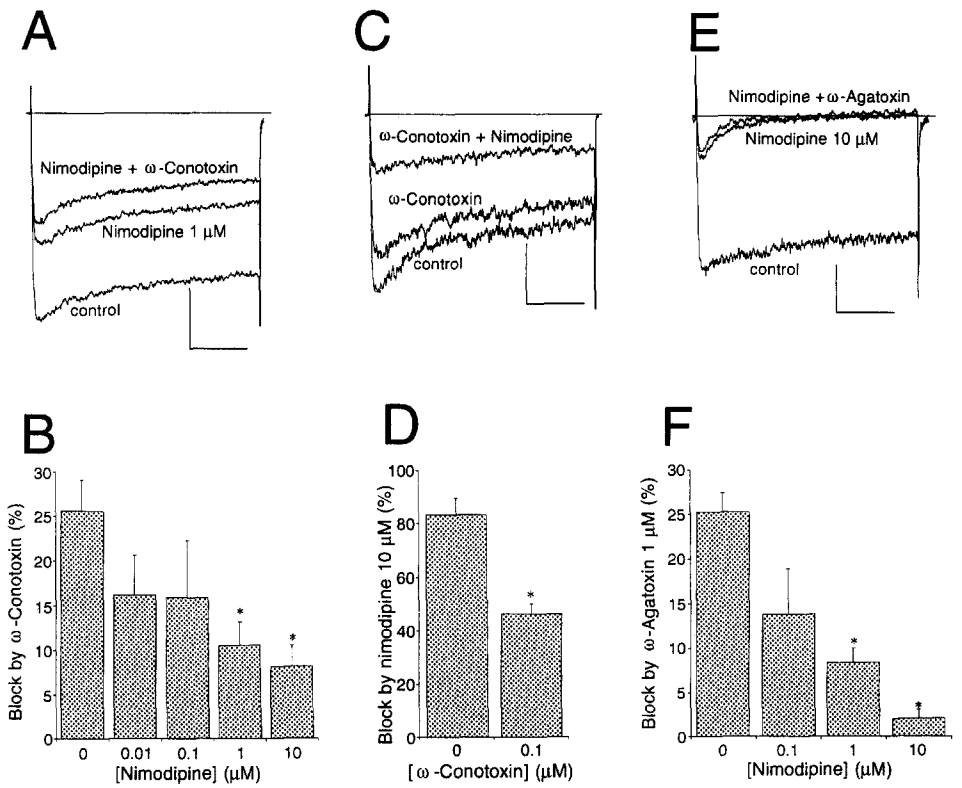

Fig. 10. Block of ω-agatoxin IVA and ω-conotoxin GVIA sensitive currents by high concentrations of nimodipine. (A) Example of an experiment with 1 μM nimodipine and 1 μM ω-conotoxin GVIA. After several control traces, 1 μM nimodipine was applied. When the amount of block by nimodipine did not increase further, 1 μM ω-conotoxin GVIA, together with 1 μM nimodipine, was applied. (B) Increasing concentrations of nimodipine reduce the amount of block by 1 μM ω-conotoxin GVIA. The amount of block induced by ω-conotoxin GVIA, in the presence of the indicated concentrations nimodipine, was presented as percentage of the initial control current (where no nimodipine nor ω-conotoxin GVIA was present). Shown are averages of similar experiments as in (A) with different concentrations of nimodipine (averages of 4–12 cells). (C) Example of a reverse experiment from that in (A). Now the control was followed by application of 0.1 μM ω-conotoxin GVIA and subsequently of 10 μM nimodipine, together with ω-conotoxin GVIA. (D) ω-Conotoxin GVIA at 0.1 μM also reduces the block by 10 μM nimodipine significantly (averages of 10 (0) and 3 (0.1) cells). (E) Similar experiment as in (A), but here we compare the block by nimodipine with the block by 1 μM ω-agatoxin IVA together with 10 μM nimodipine. (F) Increasing concentrations of nimodipine reduce the amount of block by 1 μM ω-agatoxin IVA (average of 3–9 cells). Scale bars A: horizontal 50 ms, vertical 100 pA. (C,E) Horizontal 50 ms, vertical 50 pA. Significant differences between experimental conditions and controls are indicated by asterisks ($P < 0.01$; unpaired Student's t-test).

affected the block by ω-conotoxin GVIA in a concentration-dependent manner. The block by 1 μM ω-conotoxin GVIA in the presence of 10 μM nimodipine was 8.1 ± 2.4%, which was significantly lower than the 25.5 ± 3.6% in the absence of nimodipine ($n = 6$; $P < 0.01$ unpaired Student's t-test). Thus, 10 μM nimodipine blocked 70% of the ω-conotoxin GVIA-sensitive current. It is estimated from these data that nimodipine blocks 50% of the ω-conotoxin GVIA-sensitive current at a concentration of about 300–500 nM. This is well in line with $EC_{50}(2)$ of the dose response curve of nimodipine (Fig. 9B). The block of the high voltage-activated current by nimodipine was likewise affected by the presence of ω-conotoxin GVIA (Fig. 10C). Fig. 10D shows that 10 μM nimodipine blocked the high voltage-activated current by 83.2 ± 6.3% in the absence of ω-conotoxin GVIA, in contrast to 46.2 ± 4.0% in the presence of 100 nM ω-conotoxin GVIA ($n = 3$; $P < 0.01$). These data demonstrate that the inactivating high voltage-activated current blocked by ω-conotoxin GVIA has a considerable sensivity to the dihydropyridine nimodipine.

The susceptibility of the ω-agatoxin IVA-sensitive current to nimodipine was determined in a similar way. Nimodipine affected the amount of block of the high voltage-activated current by ω-agatoxin IVA (1 μM) in a concentration-dependent manner (Fig. 10E,F). While ω-agatoxin IVA caused a block of 24.9 ± 4.5% of the total current in the absence of nimodipine, this reduced to 3.1 ± 2.7% ($n = 3$) in the presence of 10 μM nimodipine (significantly different; $P < 0.01$, unpaired Student's t-test). The ω-agatoxin IVA-sensitive current was half maximally blocked by 150–250 nM nimodipine, and at a concentration of 10 μM, nimodipine blocked 90% of the ω-agatoxin IVA-sensitive current. These results indicate that also the ω-agatoxin IVA-sensitive current in melanotropic cells has a substantial sensitivity to dihydropyridines.

4. Discussion

4.1. Classification of melanotropic high voltage-activated Ca^{2+} channel currents

It is shown in this study that high voltage-activated Ca^{2+} channel currents in melanotropic cells can be classified into four groups according to their pharmacological properties. First, about 35% of the high voltage-activated current is carried by L-type channels with a very high affinity for nimodipine. These L-type channels give rise to a sustained high voltage-activated current. The EC_{50} of ~ 3 pM is in the order of magnitude of the EC_{50} of nimodipine in blocking aortic L-type channels (McCarthy and Cohen, 1989). Next, ω-conotoxin GVIA irreversibly blocked 26% of the whole cell current and this current inactivated substantially during a 200 ms testpulse, suggesting the presence of N-type-like high voltage-activated channels. However, this current was blocked for 70% by 10 μM nimodipine (see below). Furthermore, ω-agatoxin IVA reversibly blocked 31% of the total high voltage-activated current which was distinct from the ω-conotoxin GVIA-blocked current. The ω-agatoxin IVA-sensitive current was heterogenous: low concentrations of ω-agatoxin IVA (i.e. 1–10 nM) blocked a sustained component, high concentrations (0.1–1 μM) additionally blocked an inactivating component. Nimodipine (10 μM) blocked 90% of the total ω-agatoxin IVA-sensitive current, suggesting that both ω-agatoxin IVA-sensitive components are susceptible to dihydropyridine block. The heterogeneity of the ω-agatoxin IVA-sensitive current might be the result of the presence of P-type-like and Q-type-like channels, as is the case for cerebellar granule cells (Randall and Tsien, 1995). Alternatively, a single class of channels might reflect a mode-dependent ω-agatoxin IVA affinity.

There have been conflicting reports on the nature of the high voltage-activated current in melanotropes. Some studies suggested the presence of only L-type channels (Beatty et al., 1993; Wang et al., 1992), others of L- and N-type channels (Keja et al., 1992; Stack and Surprenant, 1991), and still others of L- and P-type channels (Williams et al., 1993). Our data demonstrate that melanotropes, besides classical L-type channels, express ω-conotoxin GVIA-sensitive channels and two types of ω-agatoxin IVA-sensitive channels, all of which are blocked by sub-micromolar concentrations of dihydropyridines. The presence of P- and Q-type channels in melanotropes was recently implied by the results of Ciranna et al. (1996), who showed that the dose-repsonse curve of ω-agatoxin IVA has a low and a high affinity site. However, it is not clear from their study what the kinetic properties of these currents are, nor did they examine the dihydropyridine sensitivity of these currents.

4.2. Unusual pharmacological properties of ω-agatoxin IVA and ω-conotoxin GVIA-sensitive channels

The pharmacological properties of the ω-agatoxin IVA-sensitive channels in our melanotropic cell preparation showed some striking differences from cerebellar Purkinje and granule cell P- and Q-type channels. Firstly, Mintz and Bean (1993) reported that recovery of the block of P-type channels in Purkinje cells by ω-agatoxin IVA was very slow ($\tau = 46$–56 min.), but could be accelerated drastically by applying depolarizing pulse trains (+130–150 mV). In cerebellar granule cells, block of P- and Q-type channels reversed with a time constant of 2–4 min, but the recovery was only partial (up to 23%) (Randall and Tsien, 1995). In contrast, recovery of block of the high voltage-activated current by ω-agatoxin IVA in melanotropes occurred rapidly and was almost complete in ~ 5 min.

Secondly, almost the total ω-agatoxin IVA-sensitive current in melanotropes can be blocked by 10 μM ni-

modipine. This contrasts sharply with P-type channels in Purkinje neurones, hippocampal CA1 and CA3 cells, (Llinás et al., 1992; Mintz and Bean, 1993; Usowicz et al., 1992) and P- and Q-type channels in cerebellar granule cells (Randall and Tsien, 1995), where dihydropyridine concentrations of 5–10 μM had no effect on P- and Q-type currents. However, there have been some reports on the block of P-type currents by dihydropyridines. For instance, Pearson et al. (1995), in contrast to Randall and Tsien (1995), found that ω-agatoxin IVA blocked a component of the Ca^{2+} current which overlapped for ~90% with that blocked by dihydropyridines and ω-conotoxin GVIA in cerebellar granule cells. In addition, in neocortical neurones 50% of the P-type current is blocked by 10 μM nifedipine (Brown et al., 1994). Evidently, the sensitivity of ω-agatoxin IVA-sensitive channels to other Ca^{2+} channel blockers can differ among different cell types and under different culture conditions.

ω-Conotoxin GVIA blocked ~26% of the inactivating high voltage-activated Ca^{2+} current in melanotropes irreversibly, reminiscent of the block of N-type channels in other preparations (McCleskey et al., 1987; Aosaki and Kasai, 1989; Plummer et al., 1989). However, just as the ω-agatoxin IVA-sensitive channels in our cell culture, the ω-conotoxin GVIA-sensitive channels had a relatively high affinity of 300–500 nM for the dihydropyridine nimodipine, which is unusual for N-type channels. Although Stack and Surprenant (1991) found that the inactivating current was only reversibly blocked by ω-conotoxin GVIA (26%), they also found that this current is affected by high concentrations of the dihydropyridines Bay K 8644 and nifedipine. In conclusion, ω-conotoxin GVIA and ω-agatoxin IVA-sensitive channels in melanotropic cells display an unusual sensitivity to dihydropyridines.

4.3. Do melanotropic high voltage-activated Ca^{2+} channels contain variants of the α_{1D} subunit?

It can be argued that the effects of nimodipine on the ω-conotoxin GVIA and ω-agatoxin IVA-sensitive currents result from aselective binding of dihydropyridines to N-, P-, and Q-type channels. Aselective block by dihydropyridines of non-L-type Ca^{2+} channels and even sodium and potassium channels in various cell types have been reported. However, the EC_{50} of the aselective dihydropyridine block generally is in the micromolar range (Yatani and Brown, 1985; Valmier et al., 1991; Jones and Jacobs, 1990), which is five to ten times as high as the low affinity EC_{50} of nimodipine in melanotropic cells. The only exception is formed by T-type currents in vascular smooth muscle cell lines, which were blocked by nimodipine with an EC_{50} of 100–500 nM (McCarthy and Cohen, 1989). Nevertheless, non-L-type high voltage-activated Ca^{2+} channels are generally not affected by dihydropyridines at sub-micromolar concentrations (Jones and Jacobs, 1990; Llinás et al., 1992; Mintz and Bean, 1993; Usowicz et al., 1992; Randall and Tsien, 1995). This argues against aselective dihydropyridine effects in melanotropes.

An alternative explanation is offered by the convergence of effects of dihydropyridines, ω-conotoxin GVIA and ω-agatoxin IVA on the α_{1D} subunit (Williams et al., 1992; Birnbaumer et al., 1994; Dolphin, 1995). Oocyte expression of the α_{1D} together with α_{2b} and β_2 subunits resulted in a population of channels that was sensitive to the dihydropyridines Bay K 8644 and nifedipine (91% blocked at 5 μM) and to ω-conotoxin GVIA (54% blocked at 10 μM) (Williams et al., 1992). Block of L-type Ca^{2+} channels by ω-conotoxin GVIA was reported for chick dorsal root ganglion neurones (McCleskey et al., 1987; Aosaki and Kasai, 1989) and rat dorsal root ganglion neurones (Campbell et al., 1995), where the latter authors and Williams et al. (1992) suggested the presence of α_{1D} subunits. The effects of dihydropyridines on the ω-conotoxin GVIA-sensitive currents in chick dorsal root ganglion neurones were quantatively similar to the effects of dihydropyridines on ω-conotoxin GVIA and ω-agatoxin IVA-sensitive currents in melanotropic cells. Since the α_{1D} subunit was not only found to be expressed in brain tissue (Williams et al., 1992; Hell et al., 1993), but also in (neuro)-endocrine cells and tissues like GH_3 cells, PC12 cells, pancreas, and insulin-secreting β-cells (Yaney et al., 1992; Birnbaumer et al., 1994), the expression of the L-type α_{1D} subunit, and possible splice variants, by melanotropes might explain our results.

4.4. Discrepancies with other studies on Ca^{2+} currents in melanotropes

Single channel recordings on melanotropic cells performed in our laboratory (Keja and Kits, 1994) revealed only one conductance level of ~24 pS both for sustained and inactivating high voltage-activated Ca^{2+} channels. It was suggested that sustained and inactivating whole-cell currents resulted from modal gating properties of a single class of high voltage-activated channels, as was reported for N- and L-type channels in other preparations (Keja and Kits, 1994; Plummer and Hess, 1991; Slesinger and Lansman, 1991). In the pharmacological dissection of whole-cell Ca^{2+} currents presented here, four different types of channels are found to underly the high voltage-activated current. There are two possible explanations which might account for the discrepancies. First of all, identification of Ca^{2+} channels in single channel recordings can be obscured by overlap in conductance levels, of L-, N-, and P-type channels (Swandulla et al., 1991; Usowicz et al., 1992; Llinás et al., 1992; Caterall, 1995). The identification of channel types by conductance levels would be hampered even more, if, as we suggest, the ω-conotoxin GVIA and ω-agatoxin IVA-sensitive currents are carried by channels that contain variants of the dihydropyridine-sensitive α_{1D} subunit, which would imply the presence of multiple L-type channels in melanotropes.

Secondly, differences might very well be due to culture conditions. Keja and Kits (1994) recorded from freshly isolated cells and kept them in a minimal medium. In contrast, melanotropes used for this study were cultured for up to ten days in a serum-enriched medium. Cota (1986) reported that 6–7 days culturing of melanotropes enlarged whole-cell tail Ca^{2+} currents up to 5 times. Williams et al. (1993) attributed the absence of effects of ω-conotoxin GVIA to the fact that they used a freshly isolated intact pituitary preparation, in contrast to Stack and Surprenant (1991), who found a ω-conotoxin GVIA-sensitive inactivating high voltage-activated current in 5–7 days cultured melanotropes. Ciranna et al. (1996), however, failed to observe ω-conotoxin GVIA sensitivity in cultured melanotropes. Furthermore, Chronwall et al. (1995) found that whole-cell Ca^{2+} currents were not affected by ten days of culturing. Although no systematic changes of pharmacological properties during ten days culturing were found in our experiments, an effect of the culture conditions can not be ruled out.

In conclusion, the high voltage-activated current in melanotropic cells in our culture consists of several pharmacologically distinct high voltage-activated channel types. Calcium influx through voltage-gated channels is the major trigger for exocytosis in these cells (Thomas et al., 1990), and involvement of L-type Ca^{2+} channels has been suggested for pituitary melanotropes on grounds of the sensitivity of exocytosis to dihydropyridines (Taraskevich and Douglas, 1986). Functionally, different high voltage-activated Ca^{2+} channel types in chromaffin cells have been related to different efficacies in their coupling to exocytosis (Artalejo et al., 1994). The occurance of four high voltage-activated Ca^{2+} channel types in melanotropes suggests multiple Ca^{2+}-dependent functions, including exocytosis, each requiring specific regulation by distinct Ca^{2+} channels.

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