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Extra- and intracellular action of quaternary devapamil on muscle L-type Ca^{2+}-channels

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1 The quaternary derivative of the potent verapamil-analogue, (−)-D888, (qD888, 4-cyano-4-(3,4-dimethoxyphenyl)-*N*-[2-(3-methoxy phenyl)ethyl]-*N*,*N*,5-trimethyl-1-hexanaminium) was synthesized as a novel membrane-impermeable probe to study the localization of phenylalkylamine (PAA) effector domains on L-type Ca^{2+} channels. Channel block by qD888 was investigated in smooth muscle-like (A7r5) cells after extra- and intracellular application by use of the whole-cell configuration of the patch clamp technique.

2 Extracellularly applied qD888 inhibited Sr^{2+} (I_{Sr}) ($IC_{50}=90$ μM) and Na^+ ($IC_{50}=27$ μM) inward currents through L-type Ca^{2+}-channels mainly in a resting-state-dependent manner. Structurally closely related quaternary PAAs (e.g. D890) were ineffective after extracellular application.

3 QD888 also blocked I_{Sr} from the cytoplasmic side, as did other quaternary PAAs (D890, D575). Intracellular block was clearly dependent on channel opening, which resulted in pronounced use-dependence.

4 We conclude that qD888 blocks L-type Ca^{2+} channels not only from the intracellular side, via interaction with the classical PAA binding domain, but also from the extracellular channel surface. The properties of Ca^{2+} channel block together with previous biochemical and structural data suggest that extracellular block may be mediated by a site that also confers tonic block by quaternary benzothiazepines.

Keywords: Ca^{2+} channels; A7r5 cells; phenylalkylamine receptor; benzothiazepine receptor; quaternary devapamil

Introduction

Ca^{2+}-antagonists exert their pharmacological effects, such as vasodilatation and antiarrhythmic actions, by specifically blocking Ca^{2+}-influx through voltage-dependent L-type Ca^{2+}-channels in smooth and cardiac muscle. High affinity binding domains for these drugs are located on the α_1-subunit of the channel complex. The α_1-subunit, which also forms the ion conducting pathway, carries separate binding domains for the different chemical classes of Ca^{2+}-antagonists, i.e. nifedipine-like dihydropyridines (DHPs), diltiazem-like benzothiazepines (BTZ)/benzazepines and verapamil-like phenylalkylamines (PAAs) (Glossmann & Striessnig, 1990). Photoaffinity labelling and antibody mapping studies revealed that these domains are located within the pore-forming regions of the α_1-subunit (Catterall & Striessnig, 1992; Miller, 1992; Striessnig *et al.*, 1993). It was recently demonstrated that the blocking action of verapamil and (−)-gallopamil is substantially modulated by the β_3 Ca^{2+} channel subunit (Lacinova *et al.*, 1995). Whole-cell patch clamp experiments in mammalian cells with quaternary derivatives of these Ca^{2+}-antagonists showed that the DHP and BTZ binding domains are accessible from the extracellular channel surface (Kass *et al.*, 1991; Hering *et al.*, 1993; Seydl *et al.*, 1993). Adachi-Akahane *et al.* (1993) reported extra- and intracellular effects of a quaternary BTZ at millimolar drug concentrations. The molecular determinants of the high affinity PAA interaction domain were recently identified within the transmembrane helix IVS6 (Hockermann *et al.*, 1995; Döring *et al.*, 1996). Hescheler *et al.* (1982) found, that the quaternary PAA, D890 (quaternary gallopamil) blocked Ca^{2+}-channels in cardiomyocytes only after cytoplasmic application. As D890 was ineffective when applied extracellularly it was concluded that the PAA binding domain is accessible only from the inner mouth of the channel pore. These findings with D890 were independently confirmed by several groups in neuroendocrine, neuronal, smooth and skeletal muscle cells (Hescheler *et al.*, 1982; White *et al.*, 1986; Leblanc *et al.*, 1989; Kass *et al.*, 1991; Hering *et al.*, 1993; Seydl *et al.*, 1993). In contrast to this widely accepted finding, Wegener & Nawrath (1995) demonstrated that L-type Ca^{2+} currents in ventricular myocytes are blocked by extracellular but not by intracellular application of verapamil, gallopamil and devapamil. Thus, at present it is unclear whether other verapamil-like PAAs exclusively access their binding domain on L-type Ca^{2+} channels from the cytoplasmic side of the membrane or, like DHPs and BTZs, also from the extracellular site of the channel. In radioligand binding assays the (−)-enantiomer of D888 displays diltiazem-like binding properties (Striessnig *et al.*, 1986; Reynolds *et al.*, 1986) suggesting that this compound may also interact with the diltiazem binding side of L-type channels. This hypothesis is supported by recent SAR studies of benzazepine Ca^{2+}-antagonists (Kimball *et al.*, 1993) demonstrating that the methoxyphenyl group and the tertiary amine in the D888 molecule may mimic the critical pharmacophores of diltiazem analogues.

In whole cell patch clamp studies in intact muscle cells we demonstrate that qD888 blocks the channel not only after intracellular application but also when applied extracellularly. QD888 is therefore the first PAA Ca^{2+}-antagonist for which both extracellular and intracellular actions can be unequivocally demonstrated. The impact of our findings for the interpretation of biochemical data as well as for our general understanding of the molecular pharmacology of L-type Ca^{2+}-channels is discussed.

Methods

Synthesis of quaternary (−)-D888

(qD888, 4-cyano-4-(3,4-dimethoxy-phenyl)-*N*-[2-(3-methoxyphenyl)ethyl]-*N*,*N*,5-tri-methyl-1-hexanaminium): (−)-D888

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(15 mg) was slurried in diethyl ether (3 ml) and washed with saturated Na bicarbonate (2 × 5 ml). The Na bicarbonate solution was back-extracted with ethyl acetate, the combined organic layers filtered through magnesium sulphate and evaporated in vacuo to give the free base (13.7 mg). The free base was dissolved in chloroform (3 ml), methyl iodide (100 μl) was added, and the reaction stirred under argon for 16 h. Analytical high performance liquid chromatography (h.p.l.c.) (YMC S-3 120A ODS column, 6 × 150 mm; 0%B–100%B, linear gradient over 30 min. Solvent A: 10% MeOH, 90% H_2O, 0.2% H_3PO_4; solvent B: 90% MeOH, 10% H_2O, 0.2% H_3PO_4) showed peaks at 28.13 and 28.64 min. After the quaternarization reaction about 0.5% of tertiary D888 was still detected by h.p.l.c. Therefore this material was purified further to remove any traces of tertiary (−)-D888 by elution on an S5 YMC-ODS column (20 × 250 mm) using acetonitrile: 25 mM ammonium acetate (1 : 1) as the mobile phase. The product was lyophilized twice to remove ammonium acetate, and loaded onto a column of freshly washed HP-20 resin. The HP-20 resin was washed with 100 ml of 1 M Na iodide, the iodide salt of qD888 eluted with acetonitrile, and lyophilized to provide pure qD888 with no detectable (−)-D888 by coinjection on h.p.l.c. 1H NMR (CDCl3) :d 3.25 (6H, NMe2), 3.8 (3H, OMe), 3.9 (3H, OMe), 4.0 (3H, OMe), 3.0 and 3.6 (2H, CH2N). MS (electrospray) showed no starting material at M-14.

The pure (−)-enantiomer of qD888 was also kindly provided by Drs Kirchengast and Grünhagen (Knoll AG, Ludwigshafen, Germany) and was also subjected to h.p.l.c. purification as described above.

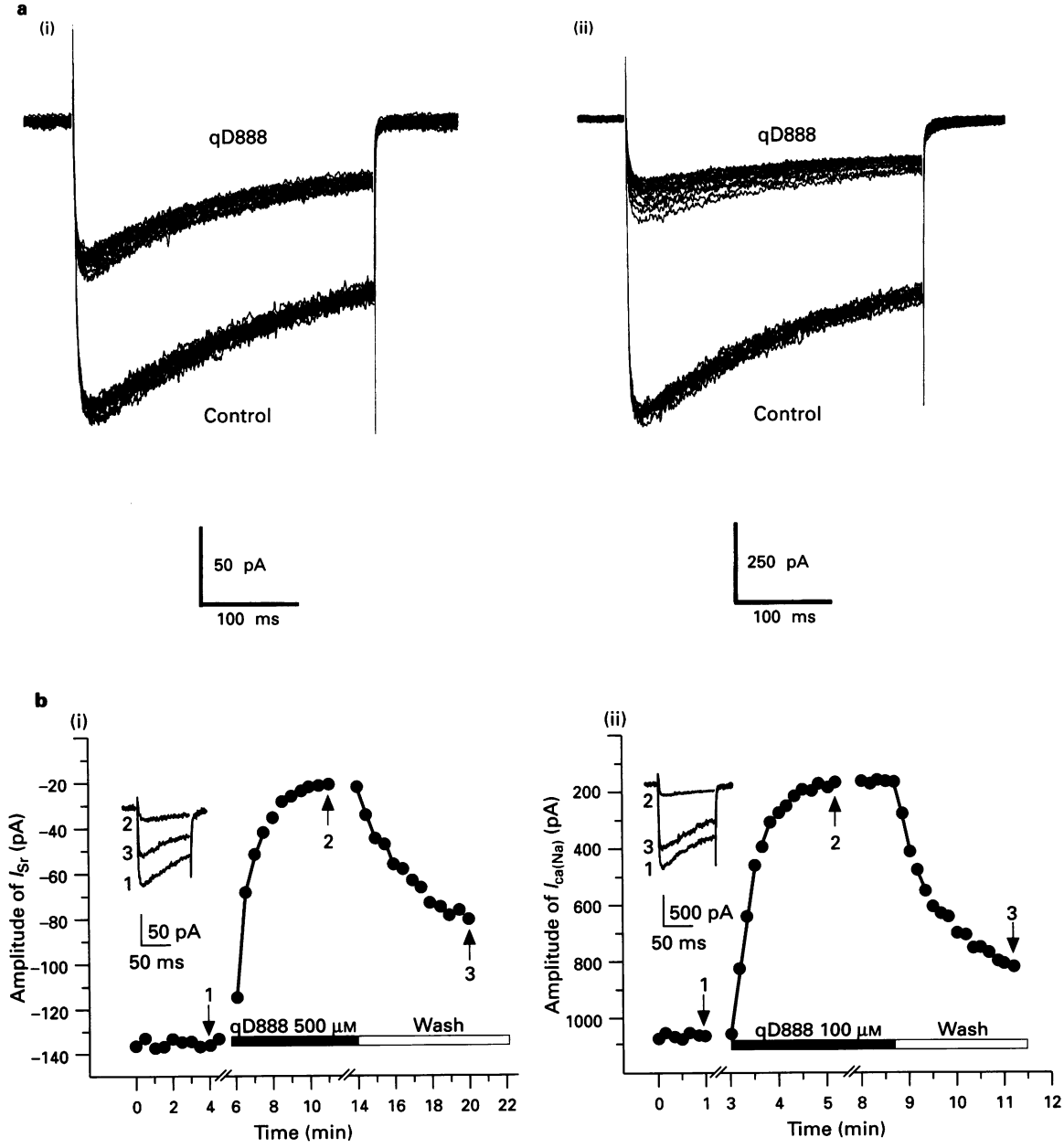

Figure 1 Resting state-dependent inhibition of I_{Sr} and $I_{Ca(Na)}$ by extracellularly applied qD888 in A7r5 cells. (a) I_{Sr} were evoked by 300-ms depolarizations from −70 mV to 20 mV (i) and sodium currents ($I_{Ca(Na)}$) from −100 mV to 0 mV (ii). Test pulses were applied at a frequency of 0.2 Hz. After application of 15 pulses in the absence of drug, the cells were incubated at rest for 5 min with either 100 μM qD888 (I_{Sr}-measurements) or 50 μM qD888 ($I_{Ca(Na)}$ measurements) followed by a pulse train in the presence of drug. (b) Reversibility of extracellular action of qD888 on I_{Sr} and $I_{Ca(Na)}$. The peak current amplitudes are plotted against time with time 0 corresponding to 2 min after establishing the whole cell configuration. I_{Sr} (i) and $I_{Ca(Na)}$ (ii) were inhibited by 500 μM or 100 μM qD888 respectively. qD888 was applied rapidly by means of a microperfusion system (see Methods) and block was partially reversed by subsequent perfusion of the cell with control solutions. Insets in (i) and (ii) show current traces recorded before (trace 1) and after (trace 2) application of the drug and after subsequent washout (trace 3).

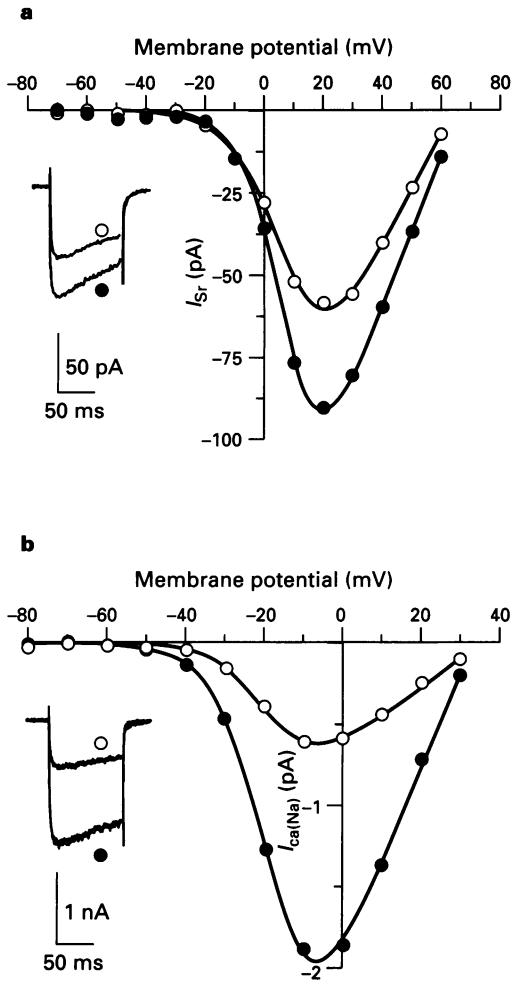

Figure 2 The effect of qD888 on the current-voltage relationship in A7r5 cells. Inward currents were evoked by 100-ms depolarizations from a holding potential of −70 mV (I_{Sr}, a) and −100 mV ($I_{Ca(Na)}$, b) before (●) and after application of 50 μM qD888 (○). Peak current values were plotted as a function of the membrane potential. Smooth lines represent the best-fit curves to the function $I_{Sr/Ca(Na)} = G_{max} * (V-V_{rev.})/(1+\exp[(V-V_{0.5,act})/k_{act}]$. $I_{Sr/Ca(Na)}$, values of the peak I_{Sr} or $I_{Ca(Na)}$ at a given test potential V; G_{max}, maximal conductance; $V_{rev.}$, reversal potential; $V_{0.5,act.}$, voltage of half-maximal current activation; k_{act} slope factor. qD888 (50 μM) decreased peak I_{Sr} and $I_{Ca(Na)}$ but the current activation threshold, the voltage of half maximal activation or the respective reversal potentials were not affected. For the experiments shown we estimated: (a, ●) $V_{0.5} = 7.8$ mV, $V_{rev} = 65.8$ mV, $k = -7.0$ mV and $G_{max} = 0.25$ nS; (a, ○) $V_{0.5} = 8.1$ mV, $V_{rev} = 64.6$ mV, $k = -7.5$ mV and $G_{max} = 0.18$ nS; (b, ●) $V_{0.5} = -17.4$ mV, $V_{rev} = 32.9$ mV, $k = -6.0$ and $G_{max} = 6.1$ nS; (b, ○) $V_{0.5} = 17.2$ mV, $V_{rev} = 32.9$ mV, $k = -6.3$ and $G_{max} = 1.8$ nS respectively. Insets (a and b): current traces from both cells recorded at +20 mV (I_{Sr}-measurements) or 0 mV ($I_{Ca(Na)}$-measurements) before and after application of 50 μM qD888.

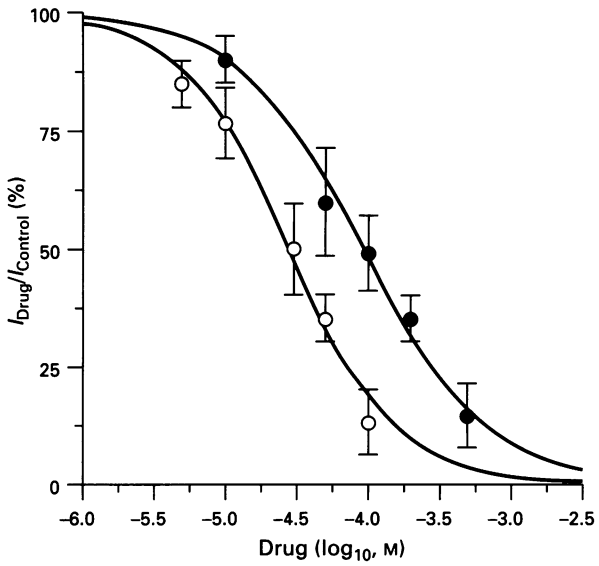

Figure 3 Concentration-dependent inhibition of I_{Sr} and $I_{Ca(Na)}$ by extracellular application of qD888 in A7r5 cells. Currents were evoked by 100-ms pulses from a holding potential of −70 mV to +20 mV (I_{Sr}) and from −100 mV to 0 mV ($I_{Ca(Na)}$). I_{drug}, maximal peak current measured in presence of the indicated drug concentration after a 'steady-state' level of peak current inhibition was reached. $I_{Control}$, maximal peak current observed before drug application. Data points were obtained from a total of 22 cells (for $I_{Ca(Na)}$, ○) and 19 cells (for I_{Sr}, ●) and are given as means ± s.e.mean (at least 3 experiments for each drug concentration). The IC_{50} values were obtained by best fit of the data points to the general dose-response equation (DeLean *et al.*, 1978): (○) $IC_{50} = 27$ μM; (●) $IC_{50} = 90$ μM.

Radioligand binding experiments

Inhibition of [^{3}H]-verapamil and (−)-[^{3}H]-devapamil binding to skeletal muscle L-type Ca^{2+}-channels by unlabelled drugs was determined as described previously (Glossmann & Ferry, 1985) at radioligand and receptor concentrations $< K_d$. Inhibition curves were fitted to the general dose-response equation to obtain IC_{50} values from which K_i values were calculated according to Cheng & Prusoff (1973).

Whole cell patch clamp experiments

Inward Sr^{2+} currents (I_{Sr}) through L-type Ca^{2+}-channels were recorded at 22–25°C by the whole cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Patch pipettes with resistances of 1 to 4 MOhm were made from borosilicate glass and filled with pipette solution. The pipette solution contained (in mM): CsCl 60, CsOH 60, aspartate 60, $MgCl_2$ 2, HEPES 10, EGTA 10 adjusted to pH 7.25 with CsOH. I_{Sr} were measured in external Sr^{2+} solution containing (in mM): $SrCl_2$ 10, N-methyl-D-glucamine 170, HEPES 10, glucose 20, 4-aminopyridine 4, tetraethylammonium chloride 27, $MgCl_2$ 3, buffered to pH 7.3 with methanesulphonic acid. Data were filtered at 2 kHz (four-pole Bessel filter). Na currents through L-type Ca^{2+}-channels ($I_{Ca(Na)}$) were measured in Ca^{2+}-free external Na solution containing (in mM): NaCl 130, HEPES 10, glucose 20, $MgCl_2$ 0.05, 4-aminopyridine 4, EGTA 2.1 buffered to pH 7.3 with NaOH. Leak conductances ranged from 0.05 to 0.3 nS in external Sr^{2+} solution and from 0.2 to 2.5 nS in external Na solution. Leak currents were subtracted digitally (using average values of scaled leakage currents elicited by a 10 mV hyperpolarizing pulse) or electronically by means of an analogue circuit. Exchange of the bath solution (i.e. external drug application) was achieved within 50 ms by means of the MPC50 microperfusion system (List electronic, Germany) as described by Savchenko *et al.* (1995). This approach enabled multiple drug applications in a single Petri dish, the precise determination of the moment of drug application as well as the start of wash out. It also substantially minimized the required amount of drug as only 50 μl was required for solution exchange in the micro-bath. For intracellular application drugs were diluted in the pipette solution and included in the patch pipette (internal dialysis) (Hering *et al.*, 1993).

Estimation of resting state-dependent and conditioned Ca^{2+} channel block

It is well established that inhibition of Ca^{2+}-channels by PAA-like Ca^{2+}-antagonists is substantially enhanced by repetitive depolarization (Lee *et al.*, 1983; Hering *et al.*, 1989; 1993; Hille, 1991). We investigated two distinct components of current

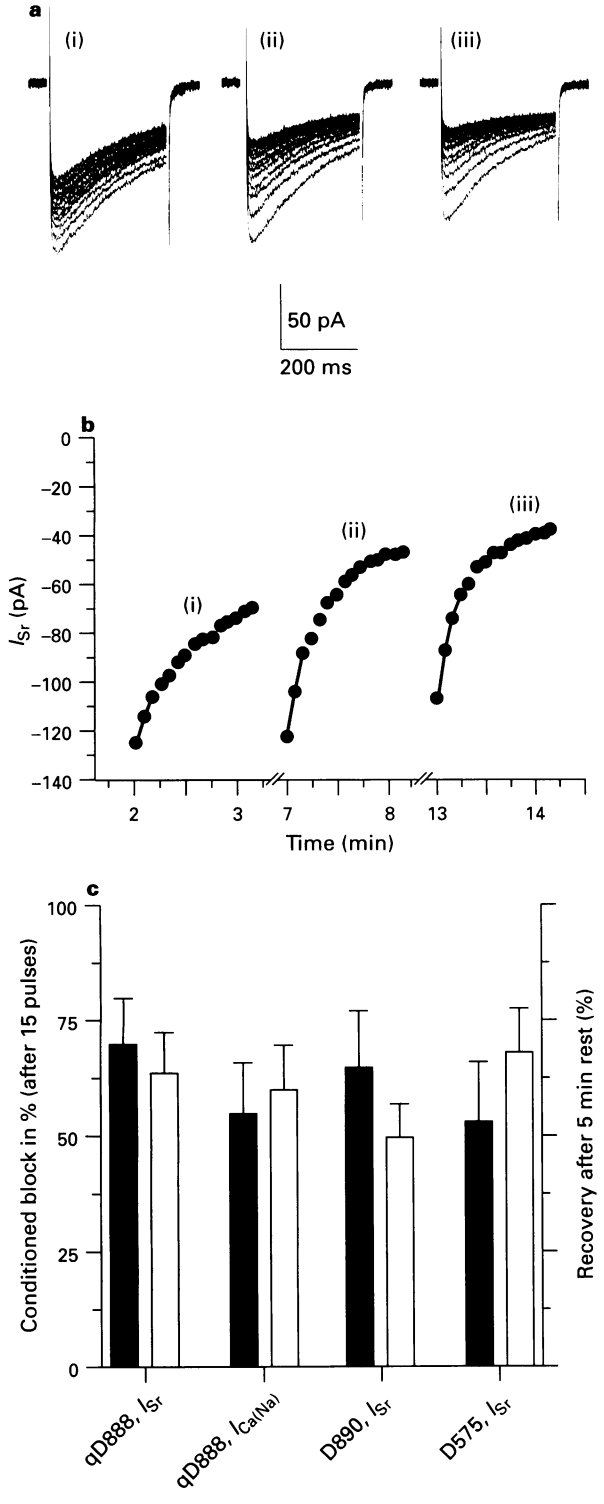

Figure 4 Intracellular action of qD888 on I_{Sr} in A7r5 cells is state-dependent. (a) I_{Sr} were elicited by 300 ms test pulses from a holding potential of −70 mV to +20 mV at the frequency 0.2 Hz. qD888 (100 μM) was included in the pipette solution. Trains of 15 pulses were applied 2 (i), 7 (ii) and 13 min (iii) after establishing the whole cell configuration. Peak current amplitudes decayed during the pulse trains (conditioned block) and almost complete recovery of I_{Sr} from block occurred during the 4 min rest between pulse trains. (b) Peak current values of the experiment shown in (a) were plotted as a function of time (X-axis breaks correspond to 4 min rest between pulses). (c) Comparison of the intracellular action of the quaternary phenylalkylamines qD888, D890 and D575 on Ca^{2+} channel currents in A7r5 cells. Conditioned block after 22 min internal perfusion with 100 μM of the respective quaternary phenylalkylamines was estimated as current inhibition after 15 pulses (same protocol as in a) and is shown as the solid columns. Test pulses were applied from −70 mV to +20 mV (I_{Sr} measurements) and from −100 mV to 0 mV ($I_{Ca(Na)}$ measurements). The amount of rest recovery from block during a 4 min stimulation free period is shown by the open columns. Columns represent the mean ± s.e.mean of at least 5 experiments.

inhibition. The current inhibition observed during the first test pulse 5 min after drug application reflecting predominantly the drug interaction with a closed non-conducting channel conformation at a holding potential of −70 mV (or −100 mV in case of $I_{Ca(Na)}$ measurements) is called 'resting state dependent' block. An additional conditioned or 'use-dependent' block by PAAs was studied during repetitive depolarizations of the cell membrane by applying trains of 15 test pulses (100–300 ms) at frequencies ranging from 0.03 to 0.2 Hz.

Cell culture

A7r5 cells were cultured as described by Hering *et al.* (1993).

Materials

(−)-[^{3}H]-D888 ((−)-[^{3}H]-devapamil) and [^{3}H]-verapamil (specific activities: 70–80 Ci mmol^{-1}) were from New England Nuclear, unlabelled phenylalkylamines were kindly provided by Dr Traut (Knoll AG, Ludwigshafen, Germany). A7r5 cells were from the American Type Culture Collection (Rockville, MD, U.S.A.).

Statistics

Data are given as range or means ± s.e.mean for the indicated number of experiments.

Results

Quaternarization of (−)-D888 yielded qD888, which was h.p.l.c. purified to remove tertiary D888. QD888 displayed slightly higher affinity for the PAA binding domain ($K_i = 1.1 \pm 0.5$ μM and 2.7 ± 0.9 μM for the [^{3}H]-verapamil and (−)-[^{3}H]-devapamil labelled domain, respectively; $n = 3$) than the quaternary derivative of gallopamil (D890; $K_i = 4.7 \pm 1.7$ μM and 13.2 ± 1 μM, respectively; $n \geqslant 3$; Hering *et al.*, 1993).

To analyse the resting state-dependent and use-dependent (see Methods) block of I_{Sr} by extracellular qD888 we applied trains of test pulses to A7r5 cells after exposing the cells for 5 min to 100 μM qD888. Because of its permanent charge, qD888 is expected to be membrane-impermeable. It should therefore only block the channel if it can directly gain access to its binding domain from the side of application. As shown in Figure 1a(i), most of current inhibition (mean inhibition 49 ± 8%, $n = 5$) occurred in the absence of repeated pulsing. This resting state-dependent block was evident as block during the first pulse of the train as compared to the control current. Peak current inhibition during a subsequently applied train of 300 ms-pulses from −70 to 20 mV (0.2 Hz) (Figure 1b(i)) was similar in the absence (mean inhibition 5.0 ± 0.5%, $n = 6$) and presence of qD888 (8 ± 4%, $n = 4$) indicating the absence of a use-dependent block component. The rate of I_{Sr} inhibition by 500 μM qD888 and subsequent partial wash out of drug are shown in Figure 1a(i). The current run-down which occurred during 10 min in the absence of drug was 6.3 ± 3.5% ($n = 16$) of initial current. QD888 did not shift the current-voltage relationship of I_{Sr} (Figure 2a). Figure 3 illustrates that block of I_{Sr} by extracellularly applied qD888 was concentration-dependent ($IC_{50} = 90$ μM). As previously shown by Hescheler *et al.* (1982) we were unable to demonstrate significant block of I_{Sr} by extracellularly applied 100 μM D890 (corresponding to about the IC_{50} of current inhibition by qD888) under the same experimental conditions ($n = 10$, data not shown).

We have previously reported, that Ca^{2+} channel currents are more efficiently blocked by the quaternary diltiazem-like

Ca^{2+} antagonist, SQ32,428 if Na ions were used as charge carrier through Ca^{2+} channels in the absence of divalent cations (Hering *et al.*, 1993). A similar observation was made in the present study for the inhibition of Na^+ currents through L-type Ca^{2+} channels ($I_{Ca(Na)}$) by qD888. As shown in Figure 1a,b and Figure 3, $I_{Ca(Na)}$ were more efficiently blocked by qD888 than I_{Sr}. Like I_{Sr}, $I_{Ca(Na)}$ was blocked in a resting state dependent manner at negative holding potentials (Figure 1a(ii)). QD888 did not affect the threshold of $I_{Ca(Na)}$ activation, the peak potential of the current voltage relationship, or the reversal potential of $I_{Ca(Na)}$ (Figure 2b). The IC_{50} of $I_{Ca(Na)}$-inhibition by qD888 ($IC_{50} = 27$ μM, Figure 3) was about three fold lower than the IC_{50} for block of I_{Sr} (90 μM, Figure 3).

Next we investigated if qD888, like D890 (Kass *et al.*, 1991), also interacts with an intracellular PAA binding domain. Figure 4 shows the effect of intracellularly applied qD888 on I_{Sr} in A7r5 cells. 100 μM qD888 was included in the pipette solution. The block development during trains of 300 ms pulses (0.2 Hz) was determined in the same cell after 2, 7 and 13 min of internal perfusion with qD888. In contrast to the extracellular action of the drug we observed pronounced use-dependent inhibition of I_{Sr} by qD888; 20 min after establishing the whole cell configuration qD888 caused a conditioned block of $70 \pm 9.8\%$ ($n = 5$) of peak I_{Sr} during the train (Figure 4c). Typically about 65% of peak I_{Sr} recovered from block in the presence of drug (100 μM qD888) within a 5 min rest at -70 mV (Figure 4b,c). Prolongation of the intracellular perfusion time resulted in a more pronounced and faster conditioned current inhibition during a train (Figure 4b). Time constants of peak current inhibition changed from $\tau_{Block} = 40 \pm 4.5$ s ($n = 3$) after 2 min to $\tau_{Block} = 21.7 \pm 3.5$ s ($n = 4$) after 13 min and $\tau_{Block} = 11.5 \pm 1.6$ s ($n = 3$) after 22 min of intracellular application (100 μM). A further prolongation of intracellular drug application time up to 27 min did not significantly accelerate use-dependent block ($\tau_{Block} = 10.3 \pm 1.1$ ms, $n = 3$) suggesting that the intracellular concentration of qD888 reached a steady-state value after about 20 min. Similar observations were made if the quaternary PAA derivatives D890 (100 μM) and D575 (100 μM) were applied to the intracellular side of the membrane (Figure 4c). Intracellular conditioned block by 100 μM qD888 ($55 \pm 11\%$ after 20 min of perfusion, $n = 4$) was also observed when Na ions were used as the charge carrier instead of Sr^{2+} (Figure 4c).

As the time of onset of drug action cannot be determined precisely upon intracellular application no estimates for resting state-dependent block could be obtained.

Discussion

Using the novel quaternary PAA qD888 we directly demonstrate for the first time, both an extracellular and an intracellular action for a verapamil-like PAA. However, extracellular and intracellular Ca^{2+} channel block show different pharmacological and biophysical properties: First, extracellular block is selectively found for qD888 but not for structurally highly related PAAs, like D575 or D890. These compounds differ from qD888 with respect to the number of methoxy groups, respectively, on their phenyl rings.

Second, extracellular block of I_{Sr} by qD888 is not use-dependent and is almost exclusively evident as a resting-state-dependent block. This is in contrast to the intracellular block found for all quaternary PAAs, including qD888, which is mainly use-dependent (Figure 4). According to previous interpretations (Hille, 1991; Hering & Timin, 1993), this suggests that the extracellular site is readily available for block, independent of the channel state, whereas block from the cytoplasmic side requires channel opening.

The tonic block by qD888 is very similar to the block previously described for a quaternary diltiazem analogue, SQ32,428 (Hering *et al.*, 1993). This compound also blocked L-type channels from the extracellular side in a resting-state dependent manner, but was ineffective after cytoplasmic application. From these data it was concluded that the BTZ binding domain is accessible from the extracellular channel surface (Hering *et al.*, 1993; Seydl *et al.*, 1993). It is therefore possible that SQ32,428 and qD888 exert their tonic block by interacting with the same region on the extracellular side of the channel. This would suggest that qD888 has affinity for the BTZ binding domain. This interpretation is supported by other independent lines of evidence: first, the *in vitro* binding properties of (−)-D888 resemble those of (+)-*cis*-diltiazem in many respects. Like (+)-*cis*-diltiazem, (−)-D888 stimulates (or stabilizes) equilibrium binding of DHP Ca^{2+}-antagonists and *vice versa* (Striessnig *et al.*, 1986; Reynolds *et al.*, 1986). Similar properties were also described for the D888-analogue, (−)-azidopamil (Striessnig *et al.*, 1987) but not for other PAAs, such as verapamil (Goll *et al.*, 1984a,b), for whose quaternary derivative we also found no evidence for an extracellular site of action.

Second, we found that, like for SQ32,428 the IC_{50} for channel block was lower when Na^+ was used as the charge carrier instead of divalent cations (Figures 1 and 3).

Third, evidence that D888 may display affinity for the BTZ binding domain comes from recent structure-activity-relationship studies (Kimball *et al.*, 1992; 1993) with D888 and a great number of diltiazem analogues. These revealed that for diltiazem-analogues two crucial pharmacophores separated by about 0.7 nm confer high affinity for the BTZ binding domain: a single (but not more than one) methoxy substituent on the 4-phenyl ring serving as a hydrogen bond acceptor, and a basic nitrogen. In contrast to e.g. verapamil, gallopamil and their respective quaternary analogues (D575 and D890), D888 and qD888 also carry only a single methoxy substituent on one of the phenyl rings (Mannhold *et al.*, 1982; Glossmann & Striessnig, 1990; Kimball *et al.*, 1993). This methoxy group is placed at an appropriate distance from its nitrogen group such that it may mimic the pharmacologically critical pharmacophores of diltiazem analogues. It was therefore proposed that D888, but not other verapamil-like PAAs, may also act through its affinity for the BTZ binding domain (Kimball *et al.*, 1993).

The high affinity determinants for PAA block of L-type Ca^{2+} channels are mainly formed by three amino acid residues within the centre of transmembrane segment S6 in repeat IV (Hockerman *et al.*, 1995; Döring *et al.*, 1996). Our data suggest that this binding pocket can be reached by membrane-impermeable PAAs from the cytoplasmic side upon channel opening. However, quaternary verapamil and gallopamil cannot gain access to this site from the extracellular surface, even if the channel frequently passes the open conformational state. It remains to be determined if the extracellular block by qD888 and SQ32,428 also involves interaction with these three amino acids. Based on their resting-state-dependent inhibition this would indicate that they can freely gain access to these residues from the extracellular site even in the absence of channel opening.

Taken together, we describe the development of qD888 as a novel probe that should help to analyse further the molecular pharmacology of L-type Ca^{2+} channels. It will be a valuable tool for identifying structural elements responsible for the resting-state-dependent block of L-type Ca^{2+} channels from the extracellular channel surface.

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