# External Action of Di- and Polyamines on Maxi Calcium-Activated Potassium Channels: An Electrophysiological and Molecular Modeling Study

Thomas M. Weiger,* Thierry Langer,# and Anton Hermann*

*Department of Animal Physiology, Institute of Zoology, University of Salzburg, A-5020 Salzburg, Austria, and #Institute of Pharmaceutical Chemistry, University of Innsbruck, A-6020 Innsbruck, Austria

ABSTRACT In this study we compared polyamines to various diamines, and we modeled flexibility as well as hydrophobicity properties of these molecules to examine possible structural differences that could explain their external effects on the channels. The natural polyamines (putrescine, cadaverine, spermidine, spermine) and diamines increasing in $CH_2$ chain length from C2 to C12 were used to probe maxi calcium-activated potassium (BK) channels in GH3 pituitary tumor cells when applied extracellularly. In single-channel recordings we found polyamines as well as diamines up to 1,10-diaminodecane to be ineffective in altering channel current amplitudes or kinetics. In contrast, 1,12-diamino dodecane (1,12-DD) was found to be a reversible blocker, with a blocking site at an electrical distance ($z\delta$) of 0.72 within the channel. It reduced single-channel current amplitude, mean channel open time, and channel open probability. In computer simulations structural data, such as flexibility, hydration, and log *D* values, were calculated. 1,12-DD showed the largest flexibility of all diamines (minimum N-N distance 9.9 Å) combined with a marked hydrophobicity due to a 4–5 Å hydrophobic intersegment between hydrophilic ends in the molecule, as confirmed by GRID water probe maps and a log *D* value of −1.82 at pH 7.2. We propose that the amount of hydration of the molecule, more than its flexibility, constitutes an essential parameter for its ability to act as a channel blocker.

## INTRODUCTION

The major natural polyamines, such as putrescine, cadaverine, spermidine, and spermine, are ubiquitously present in procaryotic and eucaryotic cells (Tabor and Tabor, 1984; Pegg, 1986). By their chemical nature, polyamines are aliphatic amines, and are polybasic and positively charged at physiological pH. Polyamines have been found to play a role in nerve growth, nerve regeneration, survival of nerve cells, and the regulation of synaptic activity, and they were found to increase after electrical stimulation of neurons (Pegg and McCann, 1982; Bondy and Walker, 1986; Pajunen et al., 1978). There is accumulating evidence that polyamines are involved in modulating or even regulating receptor- and voltage-operated ion channel activity (for review see Scott et al., 1993) and therefore may alter electrical signaling in the nervous system. When applied to the inside of the cell membrane, polyamines are responsible for rectification properties of potassium channels (Fakler et al., 1995; Ficker et al., 1994), and we have recently shown that internal polyamines modulate the activity of maxi calcium-activated potassium (BK) channels of GH3 cells (Weiger and Hermann, 1994). We further found that in neurons of the marine snail *Aplysia californica*, polyamines suppress voltage-activated potassium outward and calcium inward currents when injected into the cells (Drouin and Hermann, 1994). If applied to the external side of the membrane, polyamines modulate NMDA (*N*-methyl-D-aspartate) receptor activity (reviewed in Rock and Macdonald, 1995), and Gomez and Hellstrand (1995) reported that spermine and spermidine block voltage-dependent calcium currents. In contrast to the enhancing effect of polyamines, 1,12-diaminododecane (1,12-DD) and other long-chain diamines that also interact with NMDA receptors at the polyamine recognition site cause a voltage-dependent block of the channels. The potency of these blockers increases as the carbon chain length of the molecules is augmented (Rock and Macdonalds, 1995; Romano et al., 1992). A similar approach has previously been used to probe the structure of $K^+$ channels by the application of molecules that are variable in chain length or residues at their ends to the cytoplasmic side (Armstrong, 1971; Nomura et al., 1990; for review see Lattore, 1994). Miller (1982) probed the sarcoplasmatic reticulum $K^+$-channel from the *trans* side by using bis-quaternary ammonium ions and concluded that long-chain molecules block the channels in a bent-over configuration.

There is little knowledge about the effects of polyamines when applied to the extracellular side of BK channels. In this study we used single-channel recordings to examine the effects of external poly- and diamines on BK channel activity, and we compared polyamines to various diamines, in particular to the NMDA reverse agonist, 1,12-diaminododecane. Furthermore, we modeled the flexibility as well as hydrophilic and hydrophobic properties of these molecules by computer simulations to examine possible structural differences that could explain their external effects on the channels.

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Address reprint requests to Dr. Thomas M. Weiger, Department of Animal Physiology, Institute of Zoology, University of Salzburg, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria. Tel.: 43-662-8044-5679; Fax: 43-662-8044-5698; E-mail: thomas.weiger@sbg.ac.at.



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## MATERIALS AND METHODS

### Cell culture

GH3 pituitary tumor cells were obtained from ECACC (European Collection of Animal Cell Cultures, Salisbury, UK). Cells were cultured at 37°C and 90% humidity in MEM (minimal essential medium) supplemented with 7% fetal calf serum and 3% horse serum. For experiments cells were split and grown on poly-D-lysine-coated coverslips used 2–4 days after seeding.

### Electrophysiology and data analysis

Electrophysiological experiments were performed as previously described in detail (Weiger and Hermann, 1994). In brief: recordings from outside-out patches were taken by using a List EPC-7, filtered with a three-pole Bessel filter (corner frequency 3 kHz), stored on digital tape, DTR 1200 (Biologic, France), and off-line sampled at 12 kHz on a personal computer with the help of pClamp software (Axon Instruments, Foster City, CA). Test solutions were applied via a perfusion system (NPI electronics, Munich, Germany). Data were analyzed with pClamp software. Single-channel current amplitudes were calculated by fitting amplitude histograms to a Gaussian distribution. The channel open probability was estimated according to $P_o = [t_o/t_{tot}]/n$, where $P_o$ is the open probability for one channel, $t_o$ is the sum of open times, $t_{tot}$ is the total recording time, and $n$ is the number of individual channels observed in the patch. For analysis of channel open time distributions, samples were collected from selected bursts exhibiting only one open level. Open time distributions were fitted to a second-order exponential with pClamp software. Each experiment was repeated at least three times, and mean as well as SEM (standard error of the mean) were calculated. Significance levels were determined by using Student's *t*-test.

### Solutions and chemicals

The standard bath solution contained (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 20 glucose, 10 HEPES (pH 7.2). The regular pipette solution contained (in mM) 140 KCl, 2 $MgCl_2$, 20 glucose, 20 HEPES, 1 EGTA, 0.88 $CaCl_2$ (pH 7.2), resulting in a free calcium concentration of $1 \times 10^{-6}$ M $Ca^{2+}$, as calculated with Equal software (Biosoft, Cambridge, UK). Poly- or diamines were added to the bath solution and the pH was corrected if necessary. For rapid solution exchange, membrane patches were held in a stream of experimental solution from a second pipette.

Media for cell culture were from Biochrom (Seromed, Berlin, Germany), and sera were from PAA (Linz, Austria); all other chemicals were from Sigma (Vienna, Austria).

### Molecular modeling

For computer modeling, molecular mechanics methods were used as implemented in the computer program SYBYL (Versions 6.0 and 6.1, 1995; Tripos Associates, St. Louis, MO). Flexibility estimations were obtained using molecular dynamics simulations measuring distances between N atoms, which can be calculated from conformation changes at room temperature. Molecules were considered fully protonated for the simulation unless stated otherwise; atom partial charges were calculated using the method of Gasteiger and Marsili (1980). To start the simulation process, a 20 ps thermal activation at 2000 K was applied to the molecules. The following 100 ps was simulated at room temperature (300 K) with time increments of 1 fs. Data were collected after stable conditions were reached. Maximum and minimum distances of the N atoms were calculated, indicating the molecules' flexibility. Hydrophilicity maps were calculated using the water probe of the GRID force field (Goodford, 1985), to explore the amount and dimensions of hydration shells surrounding the molecules in its fully protonated state, as is the case in our experimental solutions.

### Log *D* calculation

Log *D* values (which represent $pK_a$ corrected log *P* values, i.e., the logarithm of the partition coefficient of the compound between water and 1-octanol at a defined pH) of the tested compounds were calculated with the software package PALLAS (Version 1.2, 1995; CompuDrug Chemistry, Budapest, Hungary). This program is based on Rekker's (1977) collection of hydrophobic fragmental constants derived from an extended "knowledge engineering" improved set of ~1000 log *P* values in the 1-octanol/water system.

## RESULTS

### Effects of poly- and diamines on BK channels

In outside-out patches, BK channels were recorded and characterized as reported previously (Weiger and Hermann, 1994). The channel conductance was 161 ± 6.9 pS ($n = 9$) under control conditions. The addition of polyamines (cadaverine, putrescine, spermidine, or spermine) in concentrations up to 10 mM at a holding potential of +40 mV, as well as diamines from a carbohydrat chain length of 2–10 C atoms, did not significantly change the channel mean open time or the open probability (data not shown). Unitary current amplitudes were reduced by cadaverine (10% ± 2.3%; $n = 3$), spermidine (13% ± 5.1%; $n = 3$), and spermine (19% ± 5.9%; $n = 3$) at concentrations of 10 mM; putrescine had no effect. Of the other diamines examined (C2–C10), only hexandiamine and diaminopropane were found to reduce single-channel current amplitudes at a concentration of 10 mM by 9% ± 2.5%, $n = 3$, and 12% ± 0.8%, $n = 3$, respectively. In contrast, 1,12-diaminododecane, a molecule similar to putrescine (differing only in the carbohydrate chain length) when applied to the outside of the membrane blocked channels in a dose-dependent, reversible manner in much lower concentrations. It reduced the current amplitude as well as the mean channel open time and open probability (Fig. 1). The dose-response curve could be fitted to the equation

$$Y = 1 - (c^n/c^n + EC_{50}^n) \quad (1)$$

where $Y$ is the normalized response, $c$ is the concentration, $n$ is the Hill coefficient, and $EC_{50}$ is the half-maximum concentration of the drug. At a holding potential of 0 mV, the $EC_{50}$ for the reduction of the single-channel current amplitude was 4.8 mM, with a Hill coefficient of 0.8 (Fig. 2). At +40 mV, $EC_{50}$ was 0.87 mM for the block of the mean channel open time, with a Hill coefficient of 1.38, and 0.41 mM for the reduction of the channel open probability, with a Hill coefficient of 2.22. The data indicate a one-to-one interaction in terms of channel current amplitude and mean open time between the blocking molecule and the channel. The reduction of the current amplitude implicates a fast blocking mechanism due to a limited frequency response of the recording system as described originally by Fitzhugh (1983). To estimate block and unblock rates, all-point amplitude histograms were generated from current traces of 50–100 ms duration. The reduction of the single-

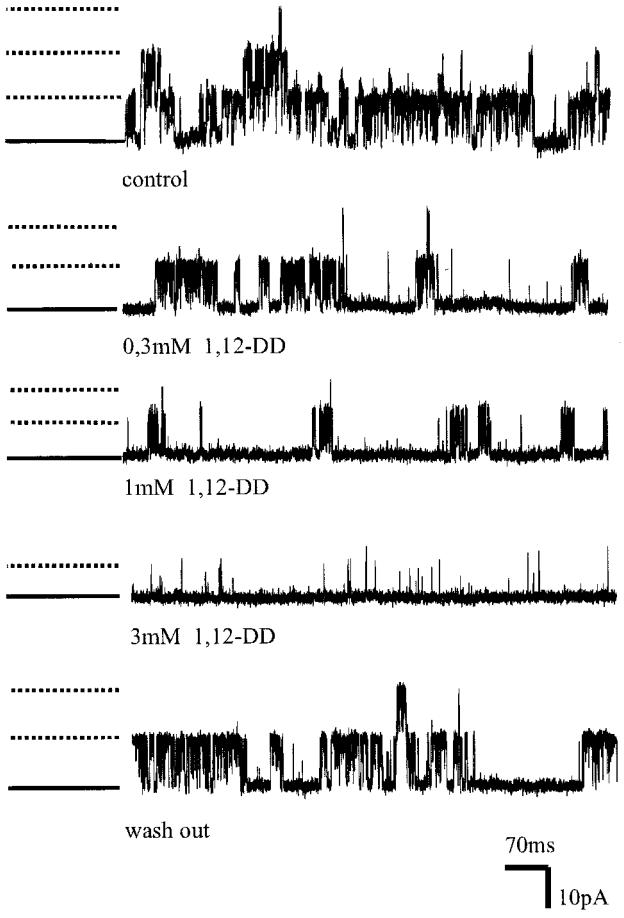


FIGURE 1 Recording of maxi calcium-activated potassium (BK) channel during control; after application of 0.3, 1, and 3 mM 1,12-diaminododecane (1, 12-DD); and after washout of the drug. ——, Closed states; ·····, first, second, and third open levels. Pipette calcium concentration 1 $\mu$M. Holding potential +40 mV.

channel current amplitude can be described by a two-state process where the open channel is seen to flicker rapidly between a blocked and an unblocked state with a unblocking rate $\alpha$ and a blocking rate $\beta$. If the current signal is passed through a first-order filter with a time constant $\tau$, the amplitude distribution of the filtered output is a $\beta$-distribution that can be described by

$$f(y) = y^{(a-1)}(1-y)^{(b-1)}/B(a,b) \quad (2)$$

where $a = \alpha/\tau$ and $b = \beta\tau$, the effective time constant $\tau = 0.228/f_c$, where $f_c$ is the $-3$ dB frequency of a three-pole Bessel filter (3 kHz in our case), and the $\beta$-function is given by

$$B(a,b) = \int_0^1 y^{(a-1)}(1-y)^{(b-1)}dy \quad (3)$$

All-point amplitude histograms from experiments with 1 mM 1,12-DD at a holding potential of +40 mV were normalized to their corresponding control values and corrected for baseline shifts. Corrected histograms were fitted to a $\beta$-distribution with the help of Prism software (Graphpad Inplot, San Diego, CA) and convolved with a Gaussian distribution fitted to the closed state of the channel (Fig. 3, *A* and *B*). The block and unblock rates obtained are shown in Figs. 3, *C* and *D*, respectively. Whereas the blocking rate, $\beta$, increased with higher concentrations of the blocker, the unblocking rate, $\alpha$, did not change significantly with increasing amounts of 1,12-DD, indicating an open channel block (Neher and Steinbach, 1978).

The amount of amplitude reduction was voltage dependent and was found to be higher at low command voltages, as shown by plotting the data according to the model of Woodhull (1973) (Fig. 4). This model describes the relative channel conductance as

$$\gamma_o/\gamma_b = \{1 + [1,12\text{-DD}]/K_d(0)\exp(z\delta FV/RT)\}^{-1} \quad (4)$$

where $\gamma_o$ is the conductance under control conditions; $\gamma_b$ is the conductance in the presence of 1,12-DD; $K_d(0)$ is the dissociation constant at zero voltage; $z\delta$ is the effective valence at the blocking site, where $z$ is the valence of 1,12-DD and $\delta$ is a measure of the voltage dependence or electrical distance of the block; $V$ is the membrane voltage; and $F$, $R$, and $T$ have their usual meanings. In its more convenient linear form, this equation is

$$\ln[(\gamma_o/\gamma_b) - 1] = \ln[1,12\text{-DD}]/K_d(0)(z\delta FV/RT) \quad (5)$$

where $z\delta$ can be determined from the slope of the graph times $RT/F$ (0.025 V) and $K_d(0)$ from the zero voltage intercept of the plot:

$$K_d(0) = [1,12\text{-DD}]/\exp(y - \text{intercept}) \quad (6)$$

From the plot in Fig. 4, the value for the effective valence was calculated to be 0.72 ($z$ is assumed to be 1; see also Discussion) and $K_d(0)$ = 4.2 mM. It is evident that the blocking effect increases as the membrane voltage is made more negative. This is expected if a cationic blocker enters the channel from the outside of the membrane, because the voltage convention defines the outside as zero voltage.

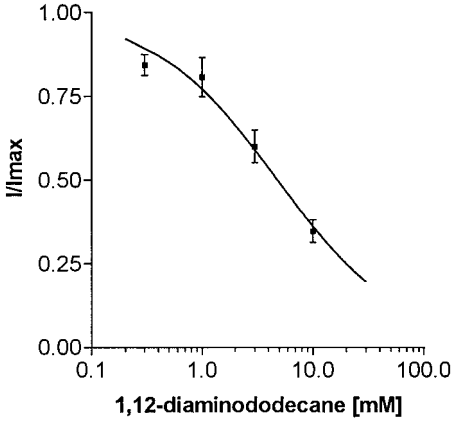


FIGURE 2 Dose-response curve of the effect of 1,12-diaminododecane on BK single-channel current amplitudes. Holding potential 0 mV, $n = 4$.

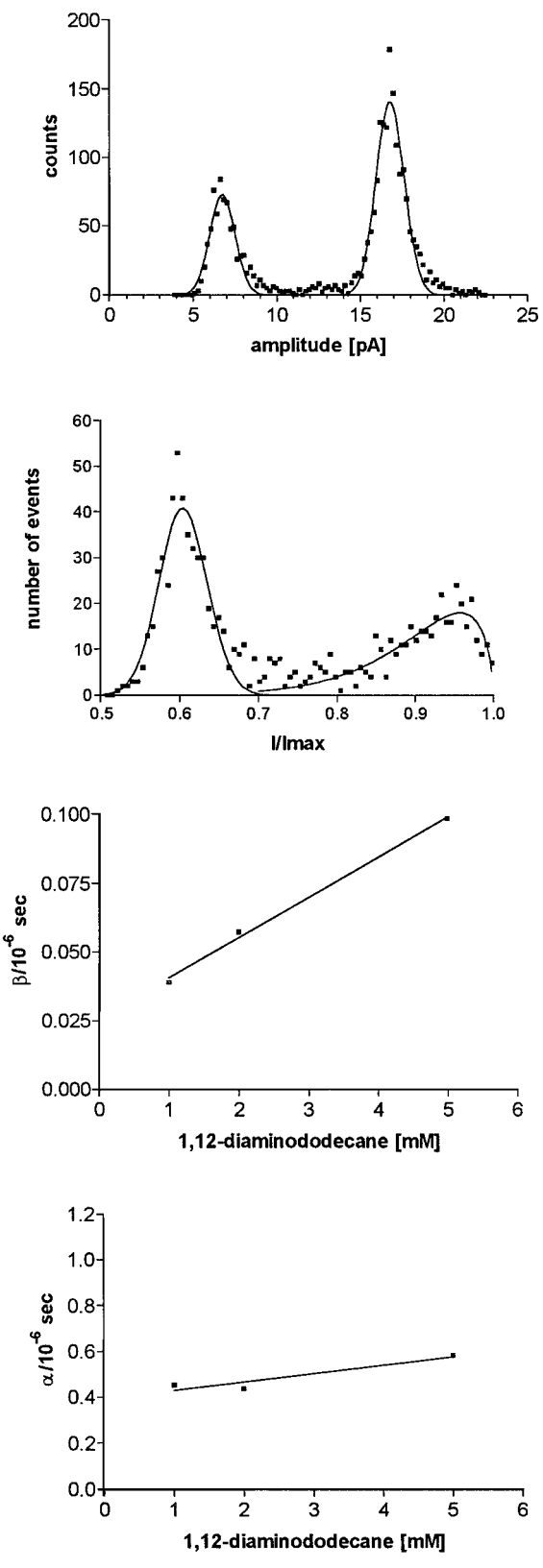


FIGURE 3 (*A*) Current amplitude distribution fitted to two Gaussians under control conditions; holding potential +40 mV. The first peak represents the closed level, and the second peak represents the open state. (*B*) Amplitude histogram of a patch held at +40 mV in the presence of 1 mM 1,12-diaminododecane. The closed level was fitted to a Gaussian curve, the broadened and skewed open level peak was fitted with a $\beta$-distribution as described in the text. (*C*) Block rate at different concentrations of 1,12-diamonododecane obtained from $\beta$-distributions at a holding potential of +40 mV, fitted to a linear regression. Each point represents the mean of two or three individual patches. (*D*) Unblock rate at different concentrations of 1,12-diamonododecane obtained from $\beta$-distributions at a holding potential of +40 mV, fitted to a linear regression. There was no signifcant deviation from zero ($p = 0.21$). Each point represents the mean of two or three individual patches.

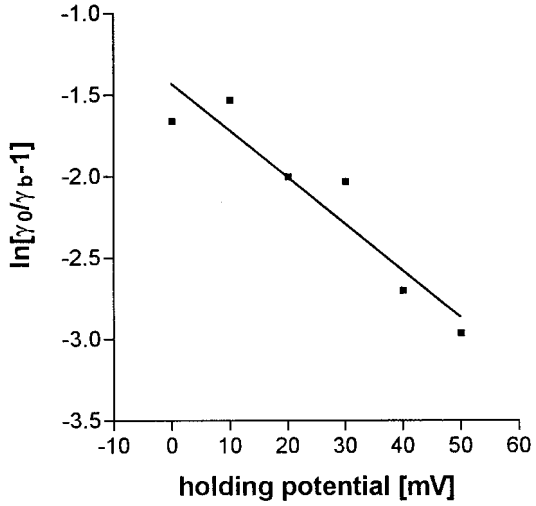


FIGURE 4 Voltage dependence of 1,12-diaminododecane (1 mM) channel block. The *y* axis represents the relation of single-channel conductance under control ($\gamma_0$) versus blocked conditions ($\gamma_b$). The solid line was fitted by regression to the linear form of the Woodhull equation as given in the text ($n = 6$).

Assuming a 1:1 reaction between the molecule and the channel pore, as the Hill coefficient from the dose-response relationship suggests, it is possible to calculate the number of sites in the channel that are not occupied by 1,12-DD:

$$D + R_b = R_bD \tag{7}$$

with a binding constant

$$K = [R_bD]/[D]\,[R_b] \tag{8}$$

where $D$ is 1,12-DD and $[R_b]$ is the concentration of the sites that can be occupied.

The total number of sites in the channel $[R_t]$ that are either occupied $[R_bD]$ or unoccupied $[R_b]$ (but, in principle, can be occupied) or can never be occupied $[R_u]$ by 1,12-DD is defined by

$$[R_t] = [R_u] + [R_b] + [R_bD] \tag{9}$$

Because the concentration of free sites $[R]$ is

$$[R] = [R_u] + [R_b] \tag{10}$$

$$([R] - [R_u])/([R_t] - [R_u]) = 1/(1 + K[D]) \tag{11}$$

and

$$[R]/[R_t] = [R_u]/[R_t] + (1 - [R_u]/[R_t])/(1 + K[D]) \tag{12}$$

Normalized channel current amplitude data obtained at +40 mV were fitted to the above equation with a $K$ of 185/M (Fig. 5). The fraction of sites that cannot be occupied by 1,12-DD ($[R_u]/[R_t]$) was 0.05, indicating that 95% of the binding sites were occupied by the molecule.

To investigate whether the observed reduction of current amplitude was artificially due to an altered gating of channels, open time distributions were analyzed. Open time distributions were fitted to a second-order exponential with two time constants ($\tau_1$, $\tau_2$). At control, $\tau_1$ was 0.73 ± 0.18 ms, and $\tau_2$ was 5.5 ± 1.2 ms ($n$ = 3 ± SEM, holding potential +40 mV). At a concentration of 0.3 mM 1,12-DD, which reduces open probablity to 74% ± 23% ($n$ = 3 ± SEM, holding potential +40 mV) of control, but affects single-channel current amplitude only sligthly, $\tau_1$ was 0.82 ± 0.21 ms and $\tau_2$ was 4.0 ± 0.82 ms ($n$ = 3 ± SEM, holding potential +40 mV). There was no statistic significant difference between control and experimental conditions.

## Molecular modeling

Molecular dimensions were calculated for 1,12-DD and tetraethylammonium (TEA). TEA had a cross-sectional diameter of 9 Å as calculated by Sybyl, whereas 1,12-DD was found to have a maximum cross-sectional diameter of 4–5 Å (Fig. 6). For comparison, potassium ions have a diameter of 2.8 Å (Marcus, 1988) (Fig. 6).

Fig. 7 shows a plot of N-N distances of 1,12-DD versus time. The molecule exhibits large oscillations in N-N distances during the first 20 ps of thermal activation at 2000 K to start the simulation process and then settles between 9.9 Å and 13.8 Å at room temperature (300 K), from which data were taken. Estimation of the flexibilities of different molecules revealed that spermine is the most flexible of the molecules tested (Fig. 8). Relative distances of $N_1$-$N_4$ were

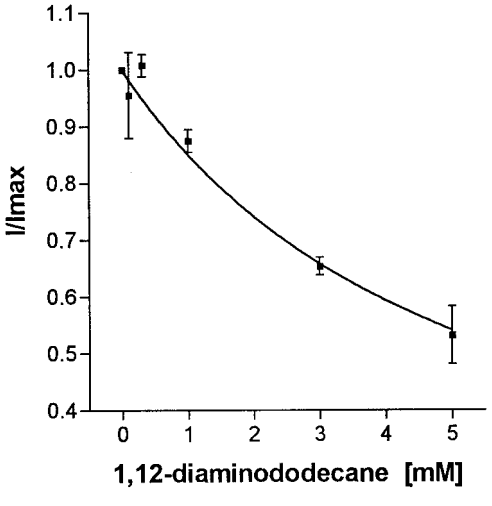


FIGURE 5 Reduction of single-channel current amplitude by 1,12-diaminododecane at various concentrations. The solid line was fitted to Eq. 12 as given in the text. Holding potential +40 mV ($n$ = 4).

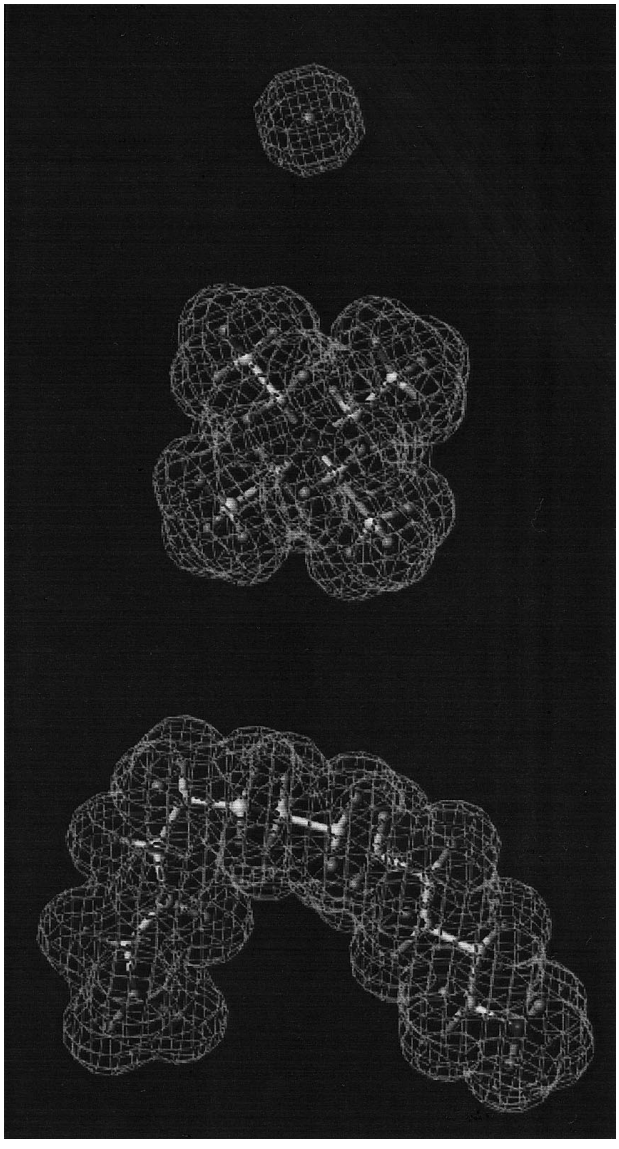

FIGURE 6 Molecular dimensions of (*top*) potassium (diameter 2.8 Å); (*middle*) tetraethylammonium (TEA) (diameter 9 Å); and (*bottom*) 1,12-diaminododecane drawn in its maximum bend-over formation with an N-N distance of 9.9 Å (cross-sectional diameter of a single amino group of 1,12-diaminododecane, 4–5 Å). Note that 1,12-diamodocecane in this configuration occupies 4.8 Å more space (maximum diameter 13.8 Å) than TEA does.

in a range from 6 Å to 13.8 Å. All other poly- and diamines had lower flexibilities, except 1,12-DD, which had a flexibility of 9.9–13.75 Å (Fig. 8). The flexibility of 1,12-DD was little changed if only one amino group was protonated (12.9–15.4 Å). To compare our calculated values with those in the literature, we also estimated flexibilities of some bis-quaternary ammonium ions, such as hexamethyldecanamine and hexamethyldodecandiamine (Fig. 8). From these results it appears that the flexibility of a molecule might play a role in easing its access to the channel pore and its binding site(s).

However, spermine, a molecule that has a flexibility similar to that of 1,12-DD, is almost ineffective in altering

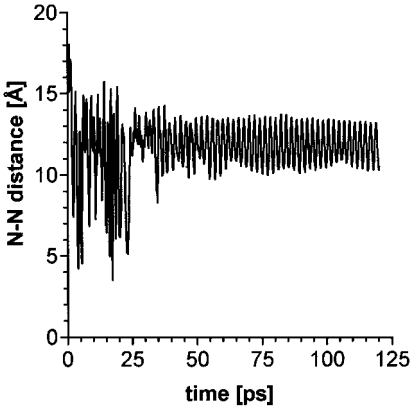


FIGURE 7 Molecular modeling: simulation of N-N distances of 1,12-diaminododecane. Note thermal activation during the first 20 ps at 2000 K (large amplitudes). The following 100 ps were simulated at room temperature (300 K). The *x* axis time is in ps ($= 10^{-12}$ s); the *y* axis N-N distance is in Å.

channel conductance or kinetics. Therefore, further differences between the molecules with respect to their blocking efficacy were expected. It is well known that the hydration of a molecule is important for its ability to enter or pass through a channel. The question concerning the hydration of polyamines and amines tested was therefore addressed by using the GRID water probe approach. As expected, the results showed that all molecules in their fully protonated state have shells of water around their positively charged amino groups (Fig. 9). Diamines with a carbohydrate chain length up to 8, including putrescine, were found to be completely surrounded by a water shell, as were spermidine and spermine, because of their additional amino groups within the molecule. Beginning with a carbohydrate chain length of 10, water shells appeared to be divided into two distinct baskets at both ends of diamine molecules according to the presence of amino groups with a hydrophobic segment in between. 1,12-DD was found to have a hydrophobic intersegment of 4.0–5.0 Å (Fig. 9). It appears possible, therefore, that differences in hydration are also involved in determining whether the molecule is able to reach a binding site within the channel pore.

We further calculated log *D* values, which provide information about the hydrophobicity of a molecule; for all naturally occurring polyamines, for 1,10-diaminodecane as well as for 1,12-DD (Table 1, negative values indicate hydrophilicity and positive values indicate lipophilicity. The results obtained at pH 7.2 and experimental concentrations of $K^+$, $Na^+$, and $Cl^-$ confirmed the simulations obtained with the GRID water probe. Hydrophilicity decreased in the order spermine > spermidine > putrescine > 1,10-diaminodecane > 1,12-DD. Hence 1,12-DD was the molecule with the least hydrophilicity at physiological pH and was found to become hydrophobic at more basic pH values, in contrast to polyamines (data not shown).

## DISCUSSION

1,12-DD, when applied from the extracellular side, was found to be an effective blocker of BK channels in GH3 cells, whereas diamines shorter than 1,12-DD as well as naturally occurring polyamines were unable to affect channel kinetics with respect to channel open probability or mean open time. These results are in agreement with earlier data showing that spermine was only effective if applied to the inside of the cell membrane but not when applied to the outside (Weiger and Hermann, 1994).

It appears possible that the action of 1,12-DD is the result of surface charge effects due to its two positive charges. In fact, there are contradictory reports about the influence of positive net charges on channel activity. Copello et al. (1991) reported that an increase in external positive charges caused by lowering pH results in a reduced activity of BK channels from gall bladder epithelial cells. On the other hand, Cornejo et al. (1989) reported that changing external pH had no effect on BK channels. In any case, it is expected that molecules with more positive charges have a greater impact on negative surface charges. Our data imply that the reduction of channel activity by 1,12-DD is unlikely to be caused by compensation of negative surface charges, be-

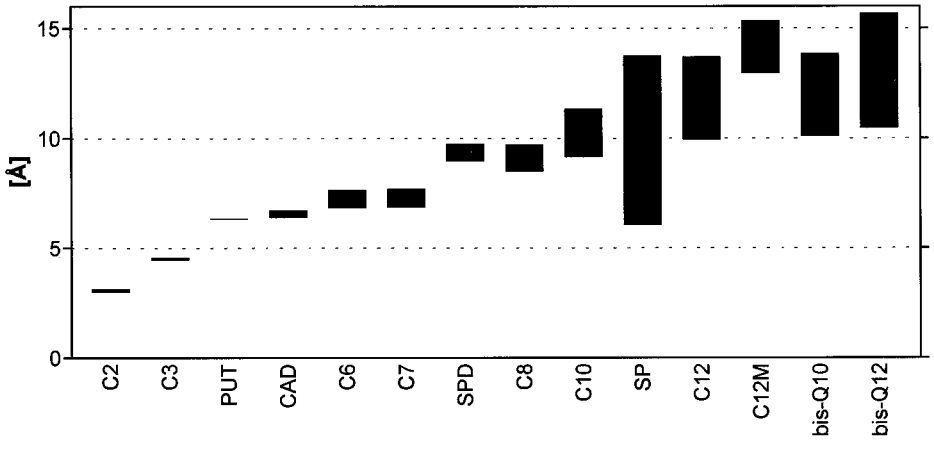


FIGURE 8 N-N distances (Å) for various molecules at 300 K (with both amino groups being protonated). C*n*, diamines with *n* C-chain length; PUT, putrescine; CAD, cadaverine; SPD, spermidine; SP, spermine; bis-Q10, hexamethyldecanamine; bis-Q12, hexamethyl-dodecandiamine. Note that C12M is 1,12-diaminododecane, with only one amino group protonated.

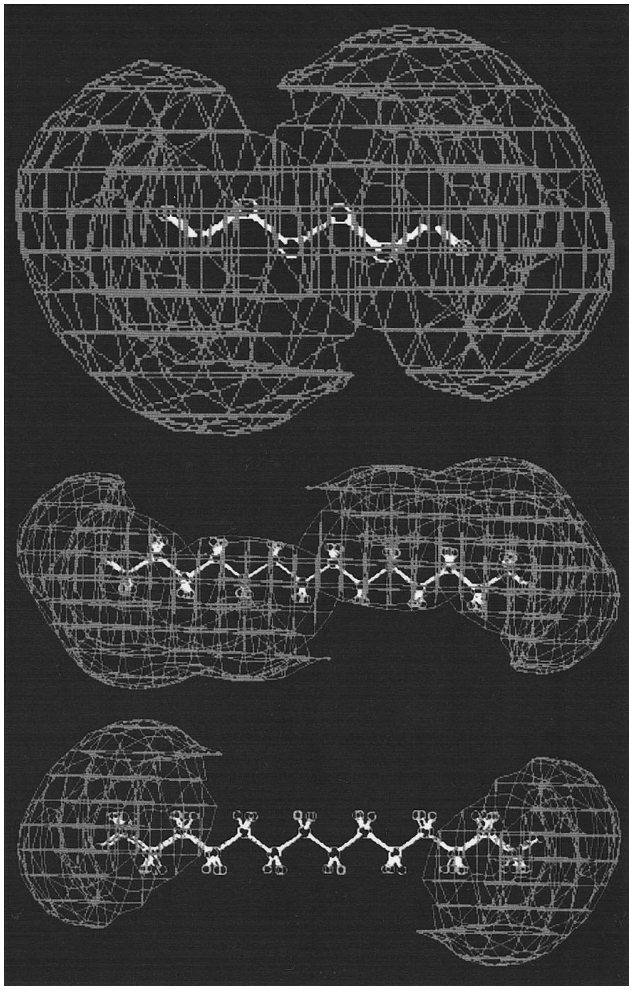

FIGURE 9 Hydrophilicity maps as calculated by the GRID waterprobe. From top to bottom: putrescine, spermine, 1,12-diaminododecane. The basket structures around the molecules indicate the hydration shells. Note the marked hydrophobic region between the terminal water baskets of 1,12-diaminododecane.

cause spermine, with four positive charges at physiological pH, was almost ineffective. Furthermore, 1,12-DD and all other diamines with two positive charges would have been expected to be about equally effective, which is not the case.

The view of a 1:1 reaction of 1,12-DD with its target inside the channel is supported by a Hill coefficient of 0.8 for the reduction of single-channel current amplitude. We further estimated that 1,12-DD occupies 95% of available binding sites. An additional, so far unknown second site of action is expected for the reduction of channel open probability. Reduction of channel open probability appears to be caused by a slow blocking mechanism. The reduction of single-channel current amplitude by 1,12-DD can be explained by a fast block, where unblock and block rates cannot be resolved by the recording system. This is in accordance with the analysis of $\beta$-distributions fitted to all-point amplitude histograms confirming a filtered two-state process of a fast flickery block. Blocking and unblocking rates are in a similar range, as previously reported by Yellen (1984) for cesium and sodium ions in this channel. Furthermore, with increasing concentrations of 1,12-DD, blocking rates were augmented, whereas the unblocking rate was not affected, as expected from an open channel block model (Neher and Steinbach, 1978). A similar combination of a fast and slow blocking mechanism has been described previously by Bokvist et al. (1990) for the action of external tetraethylammonium on calcium-activated potassium channels from mouse pancreas.

**TABLE 1 Log *D* values at pH 7.2 and $[K^+] + [Na^+] = 150$ mM, $[Cl^-] = 152$ mM corresponding to the bathing solutions used in electrophysiological experiments**

| Substance tested | log *D* |
|---|---|
| Putrescine | −5.59 |
| Spermidine | −8.34 |
| Spermine | −10.58 |
| 1,10-Diaminodecane | −2.70 |
| 1,12-DD | −1.82 |

BK channels have been shown to be permeable to ammonium ($NH_4^+$) ions (Blatz and Magleby, 1984). From dimensional considerations, this implies that the heads of diamines, which are of a size similar to that of $NH_4^+$, should be able to enter the channel. Our data, however, imply that the diameter is not the only parameter that indicates whether a diamine will act as a blocker, because variation in chain length caused considerable differences in efficacy. In the following we will discuss aspects that may help to explain the different actions of various amines in channel activity.

The finding that 1,12-DD has an effective valence, $z\delta$, of 0.72 can be interpreted in two ways. According to a model presented by Miller (1982), bis-quaternary ammonium ions, which differ from 1,12-DD only in the residue at each end of the methylene chain, bind to their target sites within the channel pore in a bend-over conformation. Here both amino groups of these molecules are able to interact with binding sites at the same electrical distance, giving effective valences of half the values calculated. Supposing a bend-over situation in the channel, it may be expected that 1) the molecule slips in its extended form into the channel, binds to one target site, and 2) bends over and attaches with its tail to a second site within the channel at the same electrical distance, i.e., close to the first amino residue. This implicates a channel dimension that gives the molecule enough space to bend over. Simulating the above situation for 1,12-DD at its closest N-N distance (9.9 Å), it is still larger in its maximum diameter (13.8 Å) than TEA (9 Å). This suggests that 1,12-DD is not able to pass the TEA binding site (at $z\delta = 0.2$) in its maximum bend-over form, because in this conformation the molecule has a diameter that is 4.8 Å larger than TEA. It is more likely, therefore, that 1,12-DD enters the channel in its extended form and then binds to a site within the channel. Once in the narrow channel pore, it appears even less likely that the second amino group of the 1,12-DD molecule is able to bend over and attach to a binding site.

It should be noted that there is a sudden increase in flexibility with 1,12-DD compared to smaller diamines. These calculations of flexibility are generally in accordance with the observations of Dufourcq et al. (1972), who investigated the conformation of dimethonium ions of various lengths by NMR. They showed methylene groups to rotate freely, but unfortunately they do not report N-N distances for longer diamines. Only spermine has a length similar to that of 1,12-DD (N-N interatomic distance of 16.024 Å versus 16.582 Å, respectively, in a fully extended, linear conformation) and therefore the capacity to almost bend over. The high flexibility of spermine is probably caused by the two N atoms in the middle of the molecule. If a bent-over conformation is involved in the blockade of channels, spermine is expected to be a prime candidate. However, spermine, irrespective of its high flexibility, is not an effective channel blocker if applied to the external side. This further supports our view that it is unlikely that 1,12-DD blocks in a bent-over confirmation. Moreover, our calculations of flexibilities for bis-quaternary ammonium ions (alkane chain $n = 10$ and 12) suggest that they are probably also too rigid to bind in a bent-over form within the narrow part of the channel. However, it is difficult to compare bis-quaternary amonium ions directly to 1,12-DD, because despite the fact that both molecules have approximately the same length, the residues are quite different in their three-dimensional size, which may lead to different binding sites of the molecules within the channel.

From our data it appears that 1,12-DD either senses only one binding site within the channel at an electrical distance of 0.72, or both positive charges bind to different sites that add up to 0.72. The latter situation predicts that shorter diamines should also be able to interact with a site located close to the channel entrance, for instance, like the extracellular TEA binding site, which has an effective valence of 0.13–0.20 (reviewed in Lattore, 1994). Our results do not support this notion, because all shorter poly- and diamines were ineffective, indicating 1,12-DD to have only one binding site within the channel, which is different from the TEA binding site. Ruppersberg et al. (1994) suggest that the effective volume including bound water molecules of a large organic blocker like argiotoxin$_{636}$ (ATX) causes an overestimation of the effective valence. This appears not to apply to our case, because 1) 1,12-DD is much smaller compared to ATX, and 2) BK channels have been shown to exhibit four binding sites for $K^+$, one of them with an electrical distance of 0.7 (Neyton and Miller, 1988), which is in excellent accordance with our findings of 1,12-DD. Furthermore, this site is also close to a $z\delta$ of 0.65 for bis-quaternary ammonium ion (bis-Q5 to bis-Q8) blockers as reported by Miller (1982) for sarcoplasmatic reticulum potassium channels. In theory, one could expect diamines of the right length as well as naturally occurring polyamines to interact with all four $K^+$-binding sites within the channel. However, because of the different dehydration energies of these molecules needed to enter the channel (see below), they appear to be excluded from interaction with these sites.

A further major difference of 1,12-DD compared to the other amines tested is its hydrophobic intersegment between hydrophilic terminal groups. It is feasible, therefore, that an interaction of the hydrophobic intersegment of 1,12-DD with a hydrophobic patch or pocket within the channel occurs. In this way the blocking action is supposed to be stabilized, as suggested by Miller (1982) for bis-quaternary ammonium blockers with an increasing $CH_2$ chain length. With respect to the amines investigated, only 1,12-DD is long and flexible enough to reach its binding site within the channel. We therefore further propose that one amino group remains hydrated within the channel vestibule outside the electrical field, which minimizes its energy needs when entering the channel's tunnel, because only one of the hydration shells on each end of the molecule has to be removed. This view is confirmed by log *D* values as well as GRID water probe simulations, indicating that 1,12-DD is the least hydrated molecule tested and therefore has the lowest energy barrier when entering the channel.

It has been suggested by several authors that $K^+$ channels are not symmetrical with respect to their inner and outer vestibules and TEA blocking sites (Hermann and Gorman, 1981; Gray et al., 1988; Villarroel et al., 1988; Lang and Ritchie, 1990; Nomura et al., 1990). The cytoplasmic side of the channel appears to be much wider than the extracellular side, which, in contrast, is more sensitive to a block by TEA and cannot be accessed by bulky ions, like the muscle relaxant gallamine (Nomura et al., 1990). The TEA binding site in BK channels of GH3 cells is at an electrical distance of $<0.2$ and has a binding constant of 260 $\mu$M when applied to the outside (Lang and Ritchie, 1990). 1,12-DD, containing an amino residue at each end of the alkyl chain, is smaller in diameter than TEA and is therefore able to slip deeper into the channel. Our finding that polyamines were ineffective up to a concentration of 10 mM when applied to the outside, as well as our previous data (Weiger and Hermann, 1994) showing polyamines to affect the channel from the cytoplasmatic side, supports the notion that this channel is not symmetrical.

External TEA is the most efficient blocker of calcium-activated $K^+$ channels in *Aplysia* neurons, whereas increasing the hydrocarbon chain length of one of the side chains decreases the binding ability of the compound (Hermann and Gorman, 1981). Villarroel et al. (1988) found externally applied bis-quaternary compounds to block BK channels with an increasing $K_d(0)$ as the methylene chain length was increased (up to five $CH_2$-groups), which was accompanied by a decrease in the effective valence $z\delta$. They interpreted these results by proposing a hydrophilic segment at the entrance of the channel, which constitutes a barrier for hydrophobic molecules. Our results show that polyamines as well as short diamines are not able to interact with the channel, even if they are more hydrophilic compared to bis-quaternary compounds. In contrast, 1,12-DD, which has a large hydrophobic intersegment, was an effective blocker. This might be due to the smaller size of the terminal amino groups compared to bis-quaternary ammonium molecules,

allowing the molecule to interact with additional sites within the channel. It is also feasible that variations in the structure of BK channels from different cells, such as a hydrophobic pouch in channels (Miller, 1982), play a role.

In conclusion, our results indicate that BK channels of GH3 cells have a blocking site for 1,12-DD at 72% of the voltage drop across the membrane when applied from the outside. All other diamines, as well as naturally occurring polyamines, are excluded from the channel, which can be explained by the distance of their charges, by their low flexibility, or most likely by the amount of hydration and hence their higher energy barrier upon entering the channel.

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