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Polyamines Block Ca^{2+}-activated K^+ Channels in Pituitary Tumor Cells (GH3)

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Abstract. The effects of the natural polyamines, putrescine, spermidine and spermine on single calcium-activated potassium channels from clonal rat pituitary tumor cells (GH3) were studied. Applied to inside-out patches, polyamines were found to reduce the current amplitude and open probability of the channels in a dose- and voltage-dependent manner, indicating that polyamines act as fast blockers which sense a fraction of the electrical field in the channel pore. The K_d for spermine was 11.2 mM for the reduction of unitary current amplitude and 0.7 mM for the reduction of the open probability. The order of effectiveness was spermine > spermidine > putrescine. From fitting β-functions to current amplitude histograms, blocking and unblocking rates were determined as 11.4×10^4 sec^{-1} and 21.9×10^4 sec^{-1}, respectively. The reduction of the channel open probability was relieved by an increase of the Ca^{2+} concentration of the internal solution, indicating that polyamines compete with Ca^{2+} at the Ca^{2+} sensor of the channel. Putrescine antagonized the effect of spermine on the channel current amplitude. The results suggest that polyamines at intracellular millimolar concentrations suppress ion channel activity and therefore may effect electrical discharge behavior of excitable cells.

Key words: Spermine — Spermidine — Putrescine — Polyamines — Ca^{2+}-activated K^+ channel — Pituitary tumor cells (GH3)

Introduction

Polyamines are polyvalent cations which are ubiquitously present in pro- and eucaryotic cells (Tabor & Tabor, 1984; Pegg, 1986; Schuber, 1989). The major cellular polyamines like putrescine, spermidine and spermine have two, three or four positive charges at physiological pH and are able to bind to a variety of anionic cellular constituents such as RNA, DNA, proteins, enzymes and membranes (Pegg & McCann, 1982; Marton & Morris, 1987; Scott, Sutton & Dolphin, 1993). A rise of intracellular polyamines up to millimolar amounts has been shown to be associated with cells actively undergoing growth, proliferation and differentiation (Pegg & McCann, 1982; Koenig, Goldstone & Lu, 1983). Tumor cells usually also contain higher amounts of intracellular polyamines than normal cells, frequently leading to the excretion of excessive polyamines which are therefore used clinically as tumor indicators (Scalabrino & Ferioli, 1985; Goldman et al., 1986; Ernestus et al., 1992).

Polyamines and ornithine decarboxylase, a major enzyme in polyamine synthesis, have been identified in the nervous tissue of a variety of tissues and species (*cf.* Shaw, 1979). Particularly after excessive electrical stimulation or after epilepsy induced by electroconvulsive shock the synthesis of polyamines is enhanced (Pajunen et al., 1978; Baudry, Lynch & Gall, 1986). Neuropharmacological actions of polyamines raise the possibility that they may modulate or mediate synaptic transmission via regulation of the free, synaptic calcium (Ca^{2+}) concentration (Bondy & Walker, 1986; Komulainen & Bondy, 1987). Polyamines also have been reported to play an essential role in nerve growth, nerve regeneration and survival of nerve cells (Gilad & Gilad, 1988; Kauppila, 1992). In a study using *Aplysia* neurons, we have shown that intracellular injection of spermine suppresses potassium outward and Ca^{2+} inward currents (Drouin & Hermann, 1990, 1994). Polyamines were found to bind to voltage-gated Ca^{2+} channels (Pullan et al., 1990; Schoemaker, 1992) and a recent report shows that external putrescine enhances L-type Ca^{2+} channel activity (Herman, Reuveny & Narahashi, 1993).

In this study we have investigated the action of in-

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ternal spermine, spermidine and putrescine on calcium-activated potassium channel (Ca^{2+}-activated K^+ channel) activity using single channel recording techniques. The results indicate that the polyamines block Ca^{2+}-activated K^+ channels by interacting at two sites, the channel pore and the Ca^{2+}-activation site. Preliminary accounts of these investigations were reported elsewhere in abstract form (Weiger & Hermann, 1991, 1992).

Materials and Methods

Clonal rat pituitary tumor cells (GH3, CCL 82.1) were obtained from European Collection of Animal Cell Cultures, UK (ECACC). Cells were used from passage 28 to 44 and cultured in Minimal Essential Medium (MEM) supplemented with 7% fetal calf serum, 3% horse serum in an atmosphere with 95% humidity and 5% CO_2 at 37°C. For experiments, cells were grown on coverslips coated with poly-L-lysine 0.1 mg/ml, MW 70,000–150,000 and used for recordings 2–4 days after splitting.

Solutions

The standard bath solution for inside-out patches with 1×10^{-6} M free Ca^{2+} contained in mM: 140 KCl, 2 $MgCl_2$, 20 glucose, 20 HEPES, 1 EGTA, 0.88 $CaCl_2$, pH adjusted to 7.2 and corrected if necessary after addition of polyamines. Free Ca^{2+} concentrations were corrected for H^+ ion and Mg^{2+} ion activity using "Equal" software (Biosoft, Cambridge, UK) and were controlled with a Ca^{2+}-selective electrode. Addition of up to 10 mM putrescine, spermidine or spermine as their chloride salts did not significantly change the free Ca^{2+} ion concentration in the experimental solutions. Measured free Ca^{2+} concentrations were: $1 \times 10^{-6.08}$ M for control solutions, $1 \times 10^{-6.1}$ M in the presence of 10 mM putrescine, $1 \times 10^{-6.15}$ M in 10 mM spermidine, and $1 \times 10^{-6.15}$ M in 10 mM spermine. Solutions with 1 mM $CaCl_2$ were not buffered with EGTA. Pipette solution in mM: 145 NaCl, 5 KCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 20 glucose, 10 HEPES, pH 7.2. For outside-out patches bath and pipette solutions were interchanged. For rapid solution exchange, membrane patches were held in a stream of the test solution from a second pipette. All chemicals were purchased from Sigma.

Electrophysiology

For experiments, cells were kept at room temperature (20–22°C) and visualized by using an inverted microscope (Leica, Labovert). Inside-out or outside-out gigaseal patches were formed and held at voltages between −20 and +30 mV (holding potential refers to the potential at the inside of the membrane). Inside-out patches were usually obtained only after brief exposure of the vesicle (which spontaneously built at the tip of the electrode) to the solution/air interface or by breaking the vesicle at the bottom of the recording chamber. Signals were amplified (List EPC-7, FRG) and low-pass filtered at 3 kHz, stored on digital tape (DTR-1200/Biologic, France) and sampled off-line at 12 kHz with pCLAMP software (Axon Instruments, Foster City, CA). For analysis of mean channel current amplitudes, the 50%-threshold method was used. Amplitude histograms were fitted to Gaussian distributions and the open and closed probabilities were calculated according to: $P_o = t_o/t_{tot}n$, where P_o = open probability for one channel, t_o = sum of open times, t_{tot} = total time of recording, and n = number of individual channels in the patch.

The channel block and unblock rates were estimated from all-points amplitude histograms using the method described by Yellen (1984). In our analysis we assumed that the reduction of the single channel current amplitude caused by polyamines can be described by a two-state process, in which the open channel flickers rapidly between blocked and unblocked states with a blocking rate β and an unblocking rate α. If the current signal is passed through a first-order filter of the time constant τ, the amplitude distribution of the filtered output is a β distribution described by the probability density function:

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

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

$$B(a,b) = \int_0^1 y^{(a-1)}(1 - y)^{(b-1)}dy \quad (2)$$

Each graph represents the mean of at least three different patches; if not indicated otherwise, bars represent the standard deviation (SD) of the mean. Means are given ± SD of at least three individual experiments where a complete set of data was obtained, unless stated otherwise.

Results

GH3 cells express various types of Ca^{2+}-activated K^+ channels which can be distinguished by their single channel conductances, their gating kinetics and their pharmacology (Ritchie, 1987; Lang & Ritchie, 1990). We have focused our studies on the "maxi" or "BK" K^+ channel which is activated by both, internal Ca^{2+}- and membrane depolarization. In asymmetrical solution, the channel has a slope conductance of 137 ± 15 pS (n = 9) (inside-out patches, *cf. control* of Figs. 1, 2), which is in the range of conductance values found for BK channels in GH3 cells by others (Dubinsky & Oxford, 1985; Rogawski, 1989) as well as in different preparations (Wong, Lecar & Adler, 1982; Smart, 1987). The channel was further characterized through its block by 1 mM external tetraethylammonium (TEA) and by low external concentrations of charybdotoxin (50 nM) in outside-out patches (*not shown*), similar to previous results obtained with pituitary tumor cells (Hermann, Erxleben & Armstrong, 1988; Lang & Ritchie, 1990; White, Schonbrunn & Armstrong, 1991).

All three types of polyamines tested (spermine, spermidine, putrescine) applied to inside-out patches suppressed Ca^{2+}-activated K^+ channel activity to various degrees (Fig. 1) and exhibited a dual effect on channels. First, with increasing concentration the open probability decreased, and second, the current amplitude of single channels was reduced. Figure 2*A* shows an example of channel activity before (*control*), after addition of spermine (2 and 10 mM) and after washout with con-

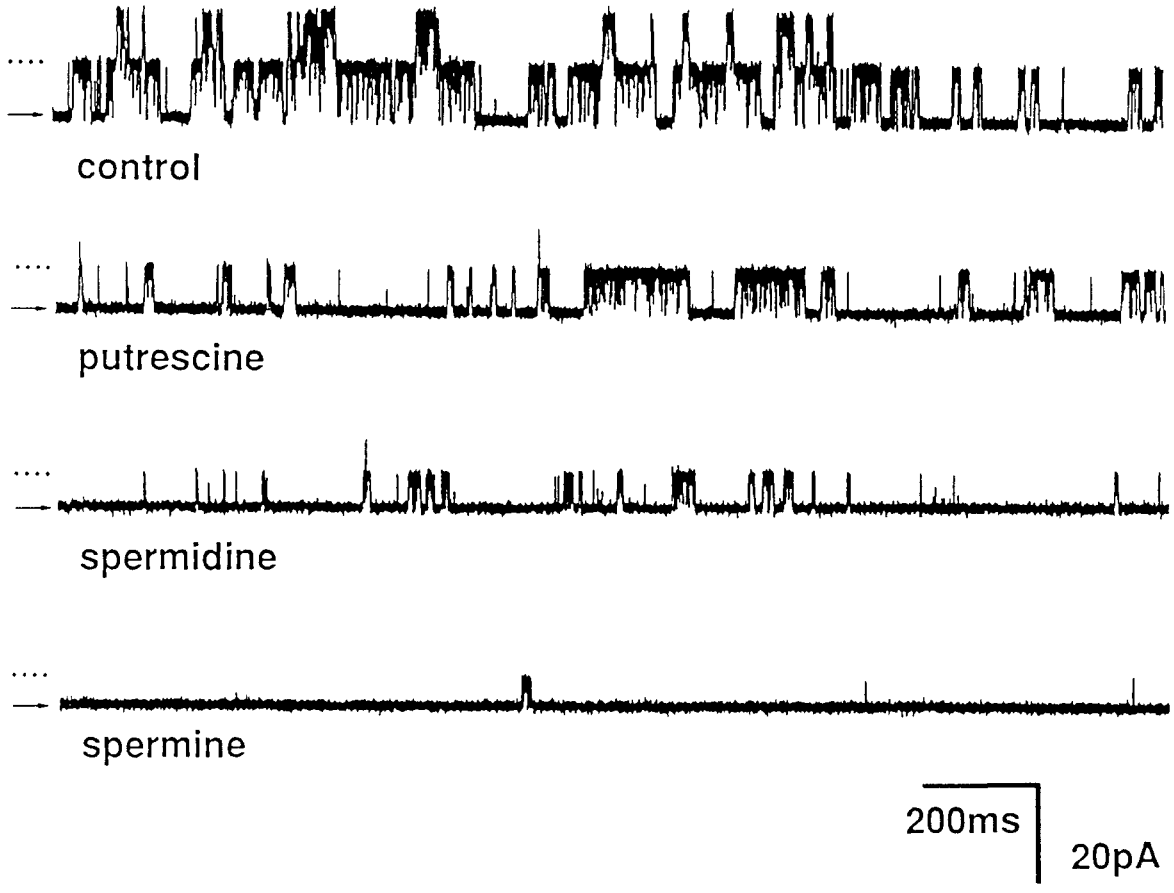

Fig. 1. Action of various polyamines on Ca^{2+}-activated K^+ channel activity. Putrescine, spermidine and spermine were applied to inside-out patches at 10 mM concentration. Holding potential +30 mV. Arrows indicate the closed states of the channels, dots indicate the first open level.

trol solution. In Fig. 2*B* amplitude histograms of the same channel are shown. Note the reduction of current amplitude and number of channel openings with increasing concentrations of spermine. Spermine was the most efficient blocking agent tested; the order of effectiveness for both current amplitude and open probability was spermine > spermidine > putrescine (Table 1). After washout, the channel current amplitude was almost restored whereas the number of channel openings remained low. The reduction of channel current amplitude may be due to the limited frequency response of the recording system resulting in a time-averaged, apparently reduced channel conductance which has been described for a number of different compounds (Yellen, 1984; Lang & Ritchie, 1990; Nomura et al., 1990). The all-points amplitude histograms obtained in the presence of 2 mM spermine at a holding potential of 20 mV were fitted by eye to a β distribution (*see* Materials and Methods) from which the rates of block and unblock of the channel were obtained. The apparent blocking rate was $11.4 \pm 4.6 \times 10^4$ sec^{-1} and the unblocking rate $21.9 \times 10^4 \pm 8.0$ sec^{-1} ($n = 3$).

In a further series of experiments, the effects of spermine were investigated in more detail. The effect of the polyamine was found to be voltage and dose dependent (Fig. 3). The efficacy of spermine in suppressing the current amplitude increased at more positive voltages; this was also true for the other polyamines tested. For example, the reduction of the single channel current in 5 mM spermine at −20 mV was 23% and at +20 mV 40%. After washout of spermine, the current was ~80% restored (Fig. 4*A*). In contrast, the open probability of the channel recovered by only ~30% after washout (Fig. 4*B*).

The dose-response behavior of the interaction of the spermine molecule, assuming a binding site in the channel, could be fitted to the four-parameter logistic equation:

$$\gamma_b/\gamma_o = A + [(B - A)/(1 + (10^c/10^X)^D)] \quad (3)$$

where γ_b and γ_o are the single channel conductances in the presence (blocked) and absence of spermine, A and B are the bottom and top plateau of the sigmoid curve,

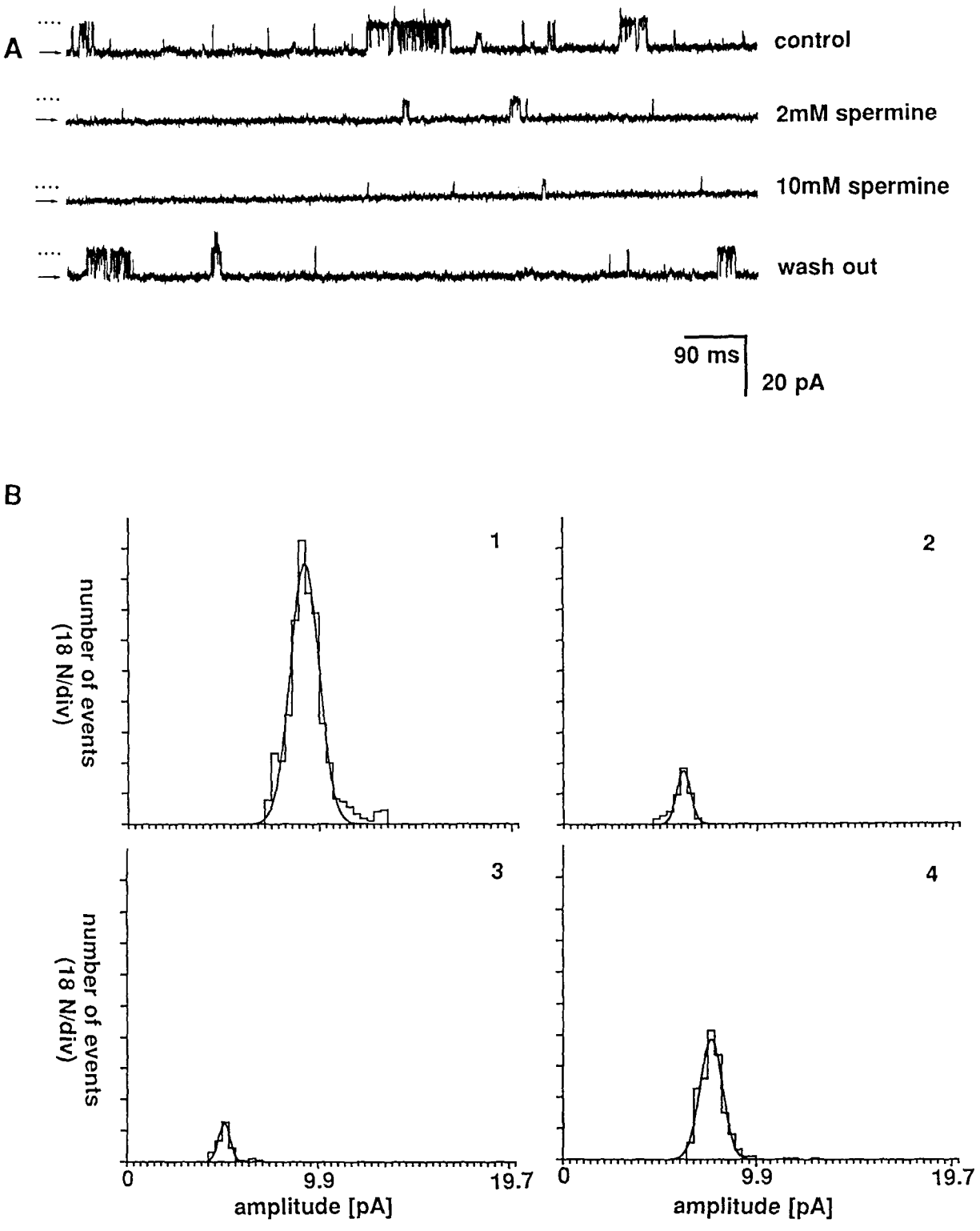

Fig. 2. Internal effects of spermine on Ca^{2+}-activated K^+ channels. (*A*) Recording of inside-out channel activity before (*control*), after application of spermine (2 and 10 mM) and after washout of spermine. Arrows mark the closed states of the channels, dots indicate the first open level. Holding potential +20 mV. (*B*) Amplitude histograms of channel currents from the same experiment. For each histogram, a 6 sec record length was analyzed. (*1*) control, mean of fit = 9.1 ± 0.7 pA, number of events = 863; (*2*) 2 mM spermine, mean of fit = 6.1 ± 0.3 pA, number of events = 86, (*3*) 10 mM spermine, mean of fit = 5.0 ± 0.3 pA, number of events = 54 and (*4*) after washout, mean of fit = 7.5 ± 0.5 pA, number of events = 289. Unbroken, bell-shaped lines represent fits of the data to a Gaussian distribution.

Table 1. Order of effectiveness of polyamines

Experimental condition	Channel current (pA)	Open probability
Control	8.4 ± 0.14	0.14 ± 0.08
Putrescine	6.1 ± 1.77	0.043 ± 0.098
Spermidine	6.0 ± 1.17	0.01 ± 0.005
Spermine	4.8 ± 0.16	0.0065 ± 0.0085

Data were obtained from histograms of single channel current amplitudes and from channel open probability at a polyamine concentration of 10 mM and compared to average control values. Holding potential +20 mV, $n = 3$.

respectively, $C = K_d$ D = Hill coefficient and X = log[spermine] (Fig. 4*A*, unbroken curve). This equation was also used to describe the concentration-dependent reduction of the channel open probability, P_o (Fig. 4*B*). The extrapolated K_d's were 11.2 ± 1.5 mM ($n = 3$) for the reduction of the channel conductance and 0.7 ± 0.4 mM ($n = 3$) for the reduction of the channel open probability at a holding potential of +20 mV. The Hill slope of 0.58 in the case of channel conductance indicates a 1:1 reaction of the spermine molecule with the channel, whereas a Hill slope of 1.57 for the open probability suggests that two molecules of spermine interact with the channel gating mechanism.

The voltage-dependent reduction of the channel amplitude indicates that the blocking molecule senses the membrane electrical field within the channel. Hence, the relative channel conductance can be described as:

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

where γ_o is the conductance under control conditions and γ_b is the conductance of the channel in the presence of spermine, $K_d(0)$ is the dissociation constant at zero voltage, $z\delta$ is the effective valence at the blocking site with z being the valency of the spermine cation and δ 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:

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

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 after $K_d(0)$ = [spermine]/exp(y-intercept). From the plot in Fig. 5, the value for the effective valency, $z\delta$, was 0.6 (if $z = 1$ is assumed, *see above and Discussion*) and $K_d(0)$ was 16.8 mM.

The differential effects of spermine on channel open probability and on channel current amplitude suggest that the polyamines act on two different sites at the channel. A possible site is the internal Ca^{2+} sensor of the channel. To test this hypothesis, spermine was applied to inside-out patches at varying bath Ca^{2+} concentrations. Under control conditions, the channel was exposed to 1 μM of free Ca^{2+} and spermine was added to the bath solution at a concentration of 2 mM at a holding potential of + 30 mV. The typical reduction of the channel open probability and the current amplitude were observed under these conditions (Fig. 6). After the free Ca^{2+} concentration was increased to 1 mM in the continued presence of 2 mM spermine, the channel open probability increased significantly but the reduction of the current amplitude remained (Fig. 6). In zero Ca^{2+}/1 mM EGTA solution, which drastically reduces channel open activity, polyamines did not alter the open probability, indicating that they were not able to act as weak agonists at the Ca^{2+} binding site (*data not shown*).

In a further series of experiments, we determined whether differently charged polyamines may compete for the same site in the channel. Inside-out patches were exposed to a solution containing 2 mM spermine followed by a solution containing both 2 mM spermine and 2 mM putrescine. Spermine caused the typical reduction of channel open activity and current amplitude. If spermine and putrescine were applied together, the open probability remained reduced, whereas the current amplitude increased but did not reach control values (Table 2). The experiment suggests that spermine and putrescine compete for the same site within the channel.

To examine the question if polyamines also act at Ca^{2+}-activated K^+ channels from the external side, outside-out patches were formed. Application of spermine at a concentration of 2 mM, which reduced channel current amplitude and open probability when applied to the inside of the membrane, had no effect compared to controls (Fig. 7).

Discussion

Our experiments show that Ca^{2+}-activated K^+ channels of pituitary tumor cells (GH3) are blocked by polyamines when applied at millimolar concentrations to the cytoplasmic side of the plasma membrane, but are ineffective if applied to the outside of the cell membrane. These observations are in accordance with studies showing that spermine blocks voltage-dependent Ca^{2+}- and K^+ conductances in *Aplysia* neurons when injected ionophoretically into the cells (Drouin & Hermann, 1990, 1994). Data obtained from receptor binding and radioactive ion flux studies also show that polyamines act on ion channels. Schoemaker (1992) recently reported that extracellular spermine and spermidine allosterically inhibit [^{3}H]nitrendipine binding to rat brain membranes and Pullan et al. (1990) showed that spermine and, less potently, spermidine inhibit the neurotransmitter-mediated ω-conotoxin-sensitive, contractile

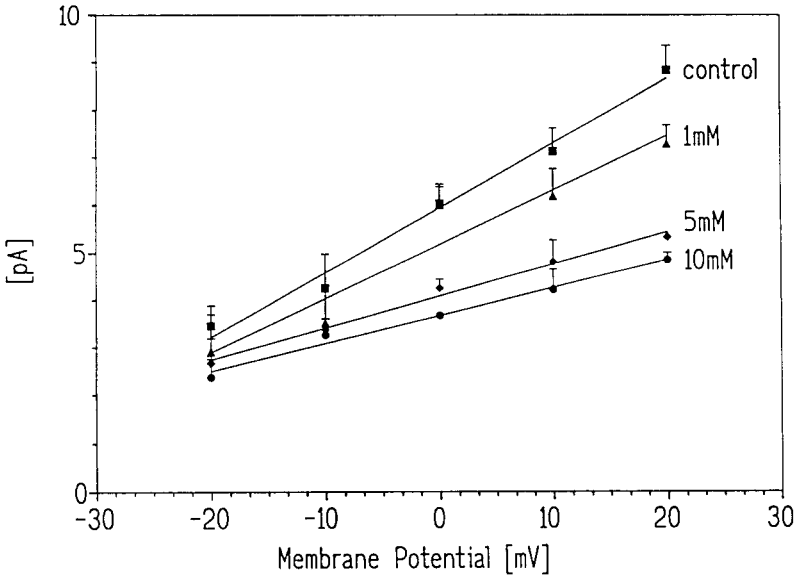

Fig. 3. Voltage- and dose-dependent effects of spermine on Ca^{2+}-activated K^+ channels. Reduction of single channel current amplitude by spermine *vs.* membrane voltage. The slope conductance of the channel is 136 pS under control conditions and 59 pS at a concentration of 10 mM spermine. Unbroken lines represent calculated regression. Vertical bars indicate standard deviation.

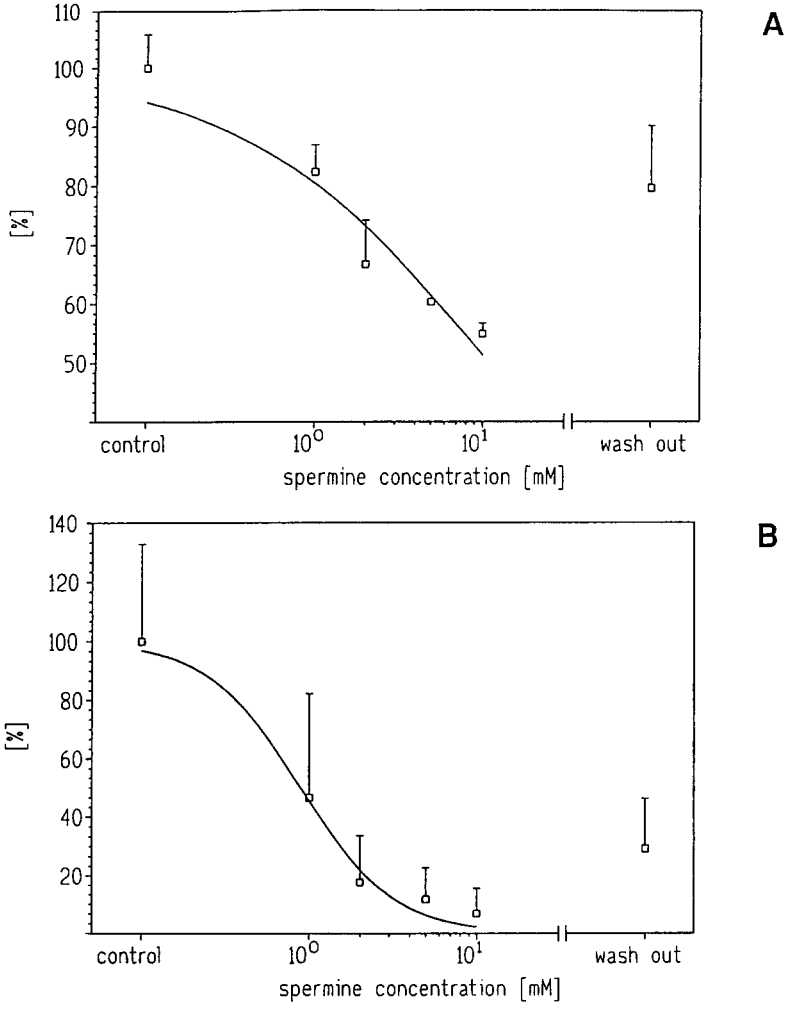

Fig. 4. Effect of spermine on Ca^{2+}-activated K^+ channel current amplitude and openings. (*A*) Dose-response plot of channel amplitudes *vs.* spermine concentration. The unbroken line drawn through the experimental points represents the calculated fit using Eq. (4), with $A = 0$, $B = 100$, $C = 10.9$, $D = 0.58$ and $X = \log[\text{spermine}]$. The channel current amplitude is restored to ~80% of the control value after washout. (*B*) Dose-response plot of channel open probability *vs.* spermine concentration. Fit of open probability at a holding potential of +20 mV using Eq. (4), with $C = 0.89$ and $D = 1.57$. The open probability was ~30% restored after washout of spermine.

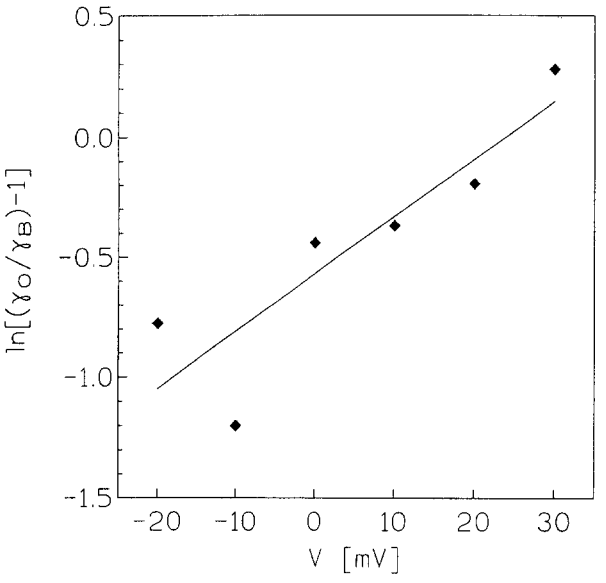

Fig. 5. Voltage dependence of spermine blockade. Plot of $\ln[(\gamma_0/\gamma_B) - 1]$ *vs.* membrane potential, where γ_o and γ_B are the single channel conductance in the absence and presence of 10 mM spermine, respectively. The unbroken line through the data points was fitted by linear regression. Each data point represents the mean of three individual experiments.

responses of rat vas deferens. In our experiments with intracellular polyamines, we found that the rank order of effectiveness at Ca^{2+}-activated K^+ channels is spermine(4+) > spermidine(3+) > putrescine(2+). This order may be assigned to the difference in positive charge or to the different chain length of the polyamines or both. Putrescine, the least potent compound, antagonizes some of the spermine effects, i.e., it partially relieves the reduction of channel current amplitude. This suggests that putrescine competes with spermine for the same binding site within the channel. Szczawinska et al. (1992) reported high concentrations of spermine in postsynaptic membrane fractions of the electric organ of *Torpedo nobiliana* where spermine at concentrations above 1 mM inhibited the ion flux at the acetylcholine receptor and competed with α-bungarotoxin binding.

It may be expected that spermine acts on internal or external negative surface charges near the channels and thus shifts activation and inactivation parameters and may decrease the local concentration of K^+ at the mouth of the channels. Assuming unspecific surface charge effects, an increased block of the channel with an increasing amount of positive charges is expected. This has been observed in studies in which the pH was lowered, i.e., by increasing the number of protons the open probability of Ca^{2+}-activated K^+ channels in rabbit tracheal smooth muscle was reduced (Kume et al., 1990). In our experiments, however, the least-charged putrescine (2+) relieved the conductance block of the higher charged spermine (4+), whereas the open probability remained unaffected. The antagonizing effect of putrescine is in accord with the report of Schoemaker (1992) showing that the effect of spermine on [^{3}H]nitrendipine binding to L-type Ca^{2+} channels in rat brain membranes was abolished in the presence of putrescine. Further indication against a major contribution of unspecific surface charge effects in our experiments are the absence of an external effect of polyamines on channel activity and the voltage-dependent nature of the block.

The blocking mechanism of the polyamines on Ca^{2+}-activated K^+ channels can be explained by assuming two sites of interaction with the channel protein: (i) the channel pore and (ii) the internal Ca^{2+} binding site. The reduction of the single channel current amplitude can be interpreted as fast block, where the channel flickers rapidly between blocked and unblocked states and due to the limited band width of the recording system results in a time-averaged reduced value of the conductance. This notion is supported by amplitude distribution analysis where β-distributions were applied to all-points amplitude histograms in a fashion consistent with a filtered two-state process of a fast flickery channel block. The rates obtained for blocking and unblocking are fast and within the range of values described by Yellen (1984) for the sodium-dependent block of Ca^{2+}-activated K^+ channels in chromaffin cells. The polyamine action is also similar to other blockers, such as the single positively charged quaternary ammonium ions (Yellen, 1984; Villarroel et al., 1988; Hu, Yamamoto & Kao, 1989; Lang & Ritchie, 1990) or the aminoglycoside antibiotics, which similarly to polyamines contain several positively charged amino residues (Nomura et al., 1990). In contrast to TEA and aminoglycosides, which have little effect on channel open and closed times, polyamines considerably altered the open/closed behavior of channels.

From the voltage dependence of the block, an effective valency, $z\delta$, of 0.6 was determined. This value of $z\delta$ is similar to that calculated for spermine blockade of *Aplysia* K^+ channels ($z\delta$ ~0.5; Drouin & Hermann, 1994) or putrescine and aminoglycoside blockade of Ca^{2+}-activated K^+ channels from rat brain synaptosomal membranes incorporated into lipid bilayers ($z\delta$ ~0.5; Nomura et al., 1990). Various models can be designed to explain these data. It is possible that the spermine molecule interacts with a single site within the channel located ~60% from its cytoplasmic orifice. Similar to a model described by Miller (1982) for bisquaternary ammonium ion block, spermine may block in a conformation with its four positive charges separated in space within the channel such that only one of its positive charges interacts with a negatively charged channel site, whereas the three other positive charges of the spermine molecule are not able to sense the electric

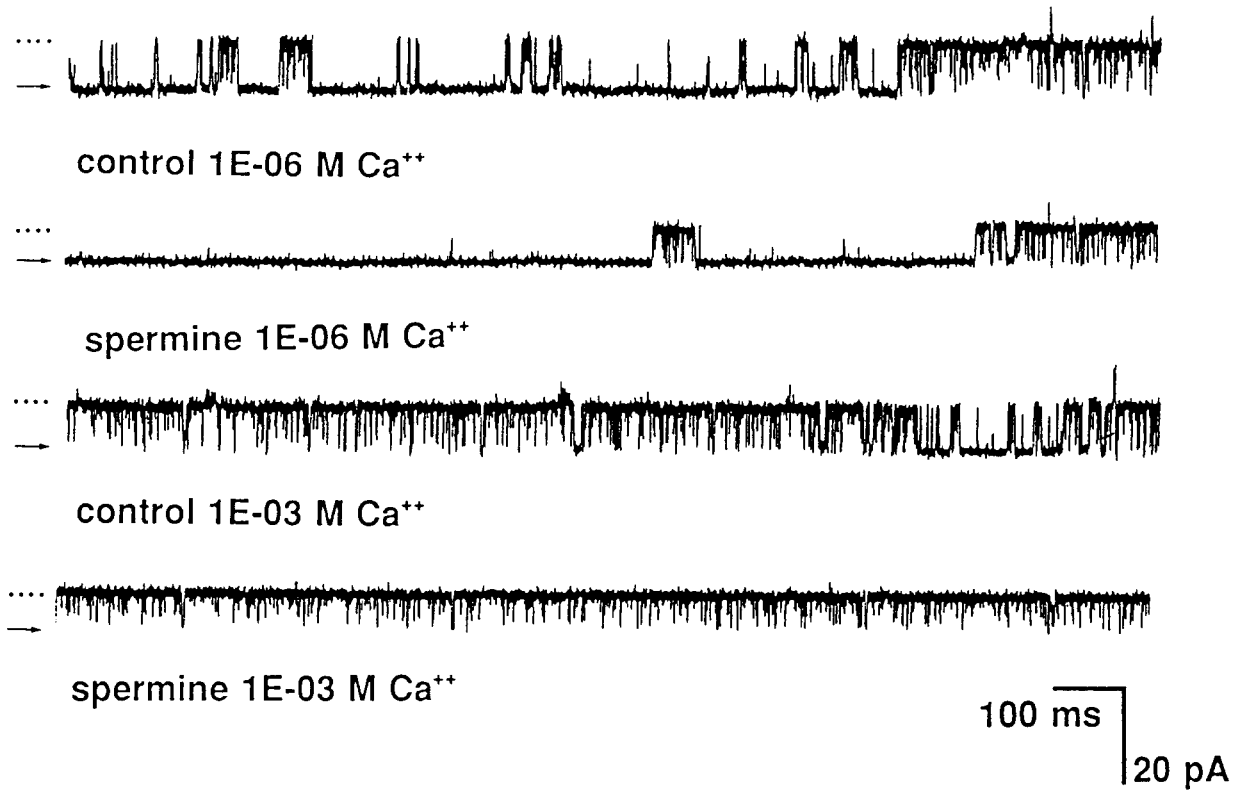

Fig. 6. Effects of Ca^{2+} and spermine on channel open probability. Inside-out single channel current records in control bath solution (top trace, 10^{-6} M Ca^{2+}) and after addition of 2 mM spermine (second trace from top). In the presence of high Ca^{2+} (third trace from top, control 10^{-3} M Ca^{2+}) channel open probability is increased and after application of 2 mM spermine in the presence of high Ca^{2+} (bottom trace) stays increased but the channel current amplitude is reduced. Single channel current amplitudes and open probabilities were 9.3 ± 0.8 pA/0.39 (2,053 events) for low Ca^{2+} control, 9.31 ± 0.65 pA/0.47 (1,244 events) for high Ca^{2+} control, 6.9 ± 0.89 pA/0.17 (375 events) in 2 mM spermine with low Ca^{2+} and 7.1 ± 0.69 pA/0.47 (1,113 events) in 2 mM spermine in the presence of high Ca^{2+} concentration. Holding potential +30 mV. Arrows indicate the closed state of the channel, dots indicate the first open level.

field. It is also feasible that two or more spermine molecules bind with one positive charge to separate sites within the channel, each sensing a fraction of the membrane voltage. This appears less likely, however, since a Hill coefficient of 0.57 indicates that one spermine molecule interacts with one site. From the block of alkylmonoamines (charge +1 and $z\delta$ of 0.25), the block of alkyldiamines of various length (charges +2 and $z\delta$ of 0.5) and the block of aminoglycosides ($z\delta$ of 0.5), it was reasoned that they share the same binding site located at 25% fractional electrical distance from the channel's internal mouth (Nomura et al., 1990). A similar shallow binding site at ~20% down the voltage drop was estimated for TEA in GH3 cells (Lang & Ritchie, 1990). It appears possible, therefore, that one spermine molecule binds in a form where its positive charges attach to various negatively charged sites in the channel and each site senses an appropriate fraction of the transmembrane electric field which adds up to a total $z\delta$ of ~0.6. This model is attractive since indeed four binding sites for K^+ ions in Ca^{2+}-activated channels from rat muscle have been found (Neyton & Miller, 1988).

Table 2. Competition of spermine and putrescine

Experimental condition	Channel current (pA)	Open probability
Spermine (2 mM)	6.4 ± 0.8	0.015 ± 0.007
Spermine + putrescine (2 mM) (2 mM)	7.5 ± 0.5	0.015 ± 0.117
Control	8.9 ± 1.2	0.045 ± 0.039

Measurements of single channel current amplitudes and open probability.

The reduction of the channel open probability and the fact that this action can be overcome by increasing the Ca^{2+} concentration, indicates that polyamines in addition act at the Ca^{2+} sensor of the channel where they may compete with Ca^{2+} for the binding site. Since polyamines are obviously able to bind, but are not able

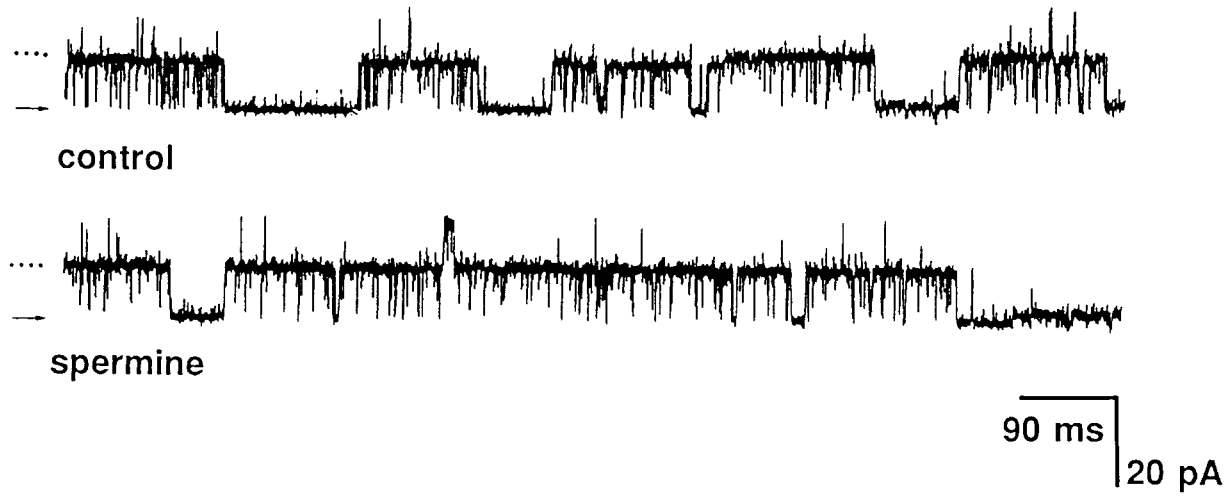

Fig. 7. External effect of spermine on Ca^{2+}-activated K^+ channels. Outside-out membrane patch in control solution (top trace) and after application of 2 mM spermine (bottom trace). Single channel current amplitudes and open probabilities of 11.18 ± 1.05 pA/0.245 ± 0.2 (n = 3) for control and 11.36 ± 0.42 pA/0.24 ± 0.14 (n = 3) for 2 mM spermine were not significantly altered. Holding potential +30 mV. Arrows indicate the closed state of the channel, dots indicate the first open level.

to open the channel, their open probability is reduced. From a fit of the data that describes the open probability *vs.* spermine concentration, a Hill coefficient of ~2 was determined indicating that two spermine molecules interact with the Ca^{2+} binding site. This is consistent with data that indicate that two or more Ca^{2+} are required for channel opening (Wong et al., 1982; Reinhart, Chung & Levitan, 1989). Our experiments also show that the channel open probability recovers only partially after washout of spermine, whereas channel conductance is almost completely reversed to control after washout. Spermine therefore may attach to the channel in a more persistent fashion, i.e., it may be associated with the protein, forming bis-(γ-glutamyl) spermine crosslinks (*cf.* Heby & Persson, 1990) and thus exert long-term modulation of the gating mechanism. Or, more likely, polyamines may function as modulators of protein phosphorylation/dephosphorylation. Large conductance K^+ channels in pituitary tumor cells are inhibited by cAMP-dependent phosphorylation and are activated by protein phosphatase activity (Armstrong & White, 1992). Indeed, polyamines have been found to enhance cAMP-dependent phosphorylation of a protein in the brain of the tobacco hornworm, *Manduca sexta,* resembling the α-subunit of voltage-sensitive Na^+ channels (Combest, Bloom & Gilbert, 1988), and spermine has been reported to inhibit cyclic nucleotide phosphodiesterase and the phosphatase calcineurin (Walters & Johnson, 1988).

In general, a block of K^+ channels results in the excitation of the cell. Polyamines, therefore may, due to their blockade of Ca^{2+}-activated K^+ channels, effect electrical properties, such as the membrane resting potential, the repolarization of action potentials, action potential frequency adaptation, regenerative discharge activity or regulation of secretion in which these channels are involved (*cf.* Hermann & Hartung, 1983; Petersen & Maruyama, 1984; Gola, Ducreux & Chagneux, 1990; Crest & Gola, 1993). Our findings suggest that the natural polyamines at intracellular millimolar concentrations, which can be obtained at a remarkably rapid rate (Koenig et al., 1983), may play an important role in controlling electrical activity of excitable cells during various physiological and pathological conditions.

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