Research Paper

Modulation of Neuronal Voltage-Activated Calcium and Sodium Channels by Polyamines and pH

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Submitted: 08/28/07
Revised manuscript submitted: 06/06/07
Manuscript accepted: 09/05/07

Previously published online as a Channels E-Publication.
http://www.landesbioscience.com/journals/channels/article/4988

KEY WORDS
polyamine, spermine, spermidine, putrescine, surface charge screening, surface potential, Gouy-Chapman, voltage-activated calcium channel, VACC, voltage-activated Na⁺ channels, VANC, superior cervical ganglion, neuron

ABSTRACT

The endogenous polyamines spermine, spermidine and putrescine are present at high concentrations inside neurons and can be released into the extracellular space where they have been shown to modulate ion channels. Here, we have examined polyamine modulation of voltage-activated Ca²⁺ channels (VACCs) and voltage-activated Na⁺ channels (VANCs) in rat superior cervical ganglion neurons using whole-cell voltage-clamp at physiological divalent concentrations. Polyamines inhibited VACCs in a concentration-dependent manner with IC₅₀'s for spermine, spermidine, and putrescine of 4.7 ± 0.7, 11.2 ± 1.4 and 90 ± 36 mM, respectively. Polyamines caused inhibition by shifting the VACC half-activation voltage (V₀.₅) to depolarized potentials and by reducing total VACC permeability. The shift was described by Gouy-Chapman-Stern theory with a surface charge density of 0.120 ± 0.005 e⁻ nm⁻² and a surface potential of -19 mV. Attenuation of spermidine and spermine inhibition of VACC at decreased pH was explained by H⁺ titration of surface charge. Polyamine-mediated effects also decreased at elevated pH due to the inhibitors having lower valence and being less effective at screening surface charge. Polyamines affected VANC currents indirectly by reducing TTX inhibition of VANCs at high pH. This may reflect surface charge induced decreases in the local TTX concentration or polyamine-TTX interactions. In conclusion, polyamines inhibit neuronal VACCs via complex interactions with extracellular H⁺ and Ca. Many of the observed effects can be explained by a model incorporating polyamine binding, H⁺ binding and surface charge screening.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are important modulators of neuronal and glial function, acting intracellularly to block inward-rectifying K⁺ channels, and AMPA-type glutamate receptors. Underlining the functional importance of polyamines is the recent observation that changes in intracellular spermine concentration mediate sensory-driven synaptic plasticity. It has been proposed that spermine and spermidine may also function as extracellular neuromodulators due to their interactions with a large number of ion channels in neurons. In support of a neuromodulatory role, polyamines are present in neurons and manufactured from decarboxylation of ornithine, are transported into synaptic vesicles, are released from brain slices by depolarizing stimuli, and may be taken up by glia from the extracellular space. Polyamines have a wide range of actions on ion channels including both facilitation and block of NMDA-activated channels, inhibition of two pore domain K⁺ channels, inhibition of voltage-activated Ca²⁺ channels (VACC), and inhibition of nonselective cation channels. Other actions of the polyamines include reduction of synaptic transmission and enhanced neuroprotection in models of ischemia and hypoxia. Block of N-type VACC has been hypothesized to underlie both these actions. We addressed this hypothesis by examining the actions of polyamines on VACC currents in rat superior cervical ganglion (SCG), which have predominantly N-type VACCs. As the IC₅₀ of polyamine inhibition of mixed neuronal VACC currents has been reported as high, we explored the actions of polyamines under a range of conditions likely to alter the positively charged polyamines and potentially increase binding affinity. This involved using physiological Ca²⁺ and Mg²⁺ concentrations (1.1 mM) to minimize confounding competition between polyamines and the ions permeating the VACC. The inhibitory actions of spermine, spermidine and putrescine were compared and described with models

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of surface charge screening with and without polyamine binding. In addition, we tested whether the inhibition of VACC by polyamines was affected by changes in extracellular pH. We also examined polyamine action on voltage-activated Na+ channels (VANCs). Spermidine and spermine indirectly modulated VANCs by inhibiting block of VANCs by tetrodotoxin (TTX) at elevated pH.

MATERIALS AND METHODS

Neuronal preparation. Recordings were made from SCG neurons prepared using methods similar to those previously described. In brief, 14- to 20-day-old rats were deeply anesthetized with isoflurane and killed by rapid decapitation. These procedures were in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by O.H.S.U. Institutional Animal Care and Use Committee. The ganglia were excised, stripped of their capsule, cut into 4 to 6 pieces and incubated with 1.5 mg ml⁻¹ of collagenase D, 1 mg ml⁻¹ trypsin, and 6 mg ml⁻¹ albumin (all Sigma) in 2 ml of modified HBSS (GIBCO). Cells were dissociated by shaking. After centrifugation, the dispersed neurons were resuspended in Earles MEM supplemented with 10% FBS, 30 mM glucose, 2 mM L-glutamine, and 50 μg ml⁻¹ penicillin-streptomycin. Neurons were plated onto glass or poly-L-lysine-coated coverslips and maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Neurons were used 1–2 days after plating.

Electrophysiology. Whole-cell recordings were made from the process-free soma of SCG neurons with 2–4 MΩ electrodes at room temperature (23–24°C). Voltage-clamp recordings were obtained using Pulse software with an Axopatch 200B (Axon Instruments, Foster City, CA) or EPC-9 (Heka Instruments Inc, MA) amplifier while the neurons were perfused at 1–3 ml/min. Series resistance was compensated by 70–85%. Currents were filtered at 2–10 kHz, then digitized at 10–20 μs per point, and leak-subtracted using a -P/4 procedure. Data were analyzed using custom macros written in Igor Pro (Wavemetrics, Lake Oswego, OR).

Solutions. Extracellular recording solution contained (in mM): 135 NaCl, 15 TEACl, 4 KCl, 1.1 CaCl₂, 1.1 MgCl₂, 10 HEPES, 10 glucose, and 0.001 TTX at pH 7.35 with NaOH. Intracellular solution contained (in mM): 120 N-methyl-D-glucamine, 20 TEAOH, 11 EGTA, 10 HEPES, 10 MgCl₂, 1 CaCl₂, 4 NaATP, 0.3 NaGTP, 14 creatinine phosphate (pH adjusted to 7.2 with methanesulfonic acid). Similar solutions have been used previously to isolate neuronal VACC currents. Test solutions were applied to the neurons by gravity feed from a nearby glass capillary. (-1 mm) which was connected to a manifold. Membrane potentials were corrected for measured liquid junction potentials. Following the addition of polyamines extracellular recording solutions were titrated to pH 7.35 by the addition of hydrochloric acid unless otherwise stated. The pH of polyamine-containing solutions was adjusted to the described values by adding hydrochloric acid or sodium hydroxide.

Analysis. The IC₅₀ was obtained by fitting the curve for each neuron using the equation:

\[ I = I_{\text{max}}/1 + ([A]/IC_{50})^H \]  
(Eqn. 1)

where I is the peak inward VACC current, Iₘₐₓ is the peak inward VACC current in the absence of polyamine, [A] is the concentration of the polyamine, and H is the Hill coefficient. The data are represented as mean ± standard error of the mean in the figures and text. Current-voltage traces were transformed to permeability-voltage plots using the Goldman-Hodgkin-Katz current equation:

\[ P = \frac{[I.RT / V (zF)^2]}{(1 - \exp(-zFV/RT)) / ([Ca^{2+}] - [Ca^{2+}]_0 \exp(-zFV/RT))} \]  
(Eqn. 2)

where V represents membrane voltage, P represents permeability, R represents gas constant, T represents absolute temperature, and F represents Faraday's constant. Permeability versus voltage data were fitted with a Boltzmann function:

\[ P = \frac{P_{\text{max}}}{(1 + \exp((V_{0.5} - V)/k))} \]  
(Eqn. 3)

RESULTS

Polyamines inhibit VACCs. Polyamines were bath applied to acutely dissociated rat SCG neurons to determine their effects on VACC currents. The VACC currents in these neurons have been well characterized as mainly N-type.²⁴-²⁶ VACC currents were isolated by blocking voltage-activated Na⁺ and K⁺ channels (see Methods). SCG neurons were voltage-clamped at -70 mV and VACC currents were activated by a family of step depolarizations increasing in 10 mV increments (Fig. 1A–C). Current-voltage plots show the peak VACC currents were inhibited by 56 ± 2% (n = 4) by spermidine (5 mM) with extracellular [Ca²⁺] and [Mg²⁺] at 1.1 mM (Fig. 1B). Higher total extracellular divalent ion concentrations have been used previously to increase the signal to noise ratio for VACC currents. Using 10 mM Ca²⁺ and 0 mM Mg²⁺ in the bathing solution we observed a 30 mV depolarizing-shift in the reversal potential, a 10 mV shift in step activating peak inward current (Fig. 1B and C), and a 2.5-fold increase in the peak inward current (data not shown). Using the higher [Ca²⁺]-containing solution the inhibition of the peak inward
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Figure 1. Spermidine and spermine inhibit VACC currents in SCG neurons. (A) VACC currents activated by step depolarization to 0 mV with 1.1 mM Ca\(^{2+}\) and Mg\(^{2+}\) in the recording solution are inhibited by 5 mM spermidine. (B) Mean I-V relationship (n = 4 neurons) in the absence and presence of 5 mM spermidine normalized to 0 mV current (1.1/1.1 mM Ca\(^{2+}\)/Mg\(^{2+}\)). (C) I-V relationship (n = 4 neurons) in the absence and presence of 5 mM spermidine normalized to the 0 mV current (Ca\(^{2+}\)/Mg\(^{2+}\):10 mM/0 mM). (D) VACC currents elicited by voltage-ramp (70 to 70 mV @ 1 mV/ms) are inhibited by spermine in dose-dependent and reversible manner (Ca\(^{2+}\)/Mg\(^{2+}\):1.1 mM/1.1 mM). Cd\(^{2+}\) (100 \(\mu\)M) also inhibited VACC currents.

VACC current by 5 mM spermidine was only 27%. To further examine the effect of polyamines we activated VACC currents using depolarizing voltage ramps from -70 mV to 70 mV (1 mV/ms), which facilitated acquisition of data over a range of voltages. Application of spermine reduced the VACC current in a reversible, dose-dependent manner (Fig. 1D). Application of Cd\(^{2+}\) (100 \(\mu\)M) blocked the vast majority of the inward current (94 ± 0.7%; n = 4) confirming that the current was carried by VACCs.

Using the same ramp voltage protocol we tested the actions of spermine, spermidine and putrescine on VACC currents. The traces in Figure 2 show the currents from three exemplar cells that were activated by voltage ramps applied with a 15 second duty cycle. The peak inward currents were inhibited by increasing concentrations of all three polyamines (Fig. 2A–C). The kinetics of inhibition were relatively fast compared with the depolarization duty cycle, and peak current amplitude decreased to a new stable level normally within 15 seconds of the change in polyamine concentration (Fig. 2D). The average polyamine dose-response curves show the peak inward VACC currents normalized against the currents recorded in the absence of the antagonists (Fig. 2E) for a total of eighteen cells. The IC\(_{50}\) were different for spermine, spermidine and putrescine (p < 0.001) and measured 4.7 ± 0.7 (n = 10), 11.2 ± 1.4 (n = 5), and 90 ± 36 (n = 3) mM, respectively (p = 0.024 for spermidine vs putrecine and p = 0.0004 for spermine vs spermidine).

VACC inhibition is mediated by both shifts in activation and decreased permeation. Spermine, spermidine and putrescine are multivalent cations with predominant valences at physiological pH of 4\(^+\), 3\(^+\) and 2\(^+\), respectively. Thus the interactions between polyamines and VACCs may depend on membrane potential. Furthermore these charges may affect the membrane surface potential, and hence channel gating, by screening negative surface charge. \(^{32,37}\) Peak inward VACC current occurred at more depolarizing voltages in the presence of spermine and spermidine (Fig. 2A and B) just as the I-V curves had shifted at higher extracellular [Ca\(^{2+}\)] (Fig. 1B and C). However the outward currents were largely unchanged (Fig. 2A–C). To determine if these observations reflected a voltage shift in activation of VACCs we examined the permeability-voltage (P-V) relationship after transforming the currents activated by voltage-ramps using equation 2. The current traces in Figure 2 were converted to P-V plots and each curve fitted with a single Boltzmann equation (Fig. 3). This analysis revealed that polyamines inhibited VACC currents by two distinct reversible and dose-dependent mechanisms. Firstly, the curves were right-shifted by increasing concentrations of polyamine (Fig. 3) and the degree of shift was greatest in the order spermine > spermidine > putrescine. Higher concentrations of spermine, spermidine, and putrescine (10 mM) demonstrated this most clearly (Fig. 3D) where the V\(_{0.5}\) shifts (ΔV\(_{0.5}\)) were 9.2 ± 0.3 (n = 10), 5.1 ± 0.6 (n = 5) and 1.2 ± 1.0 (n = 3) mV, respectively (p = 0.001 overall; p = 0.036 for spermidine vs putrecine and p = 0.001 for spermine vs spermidine). Application of the Grahame equation to the spermine data, yielded a surface charge of 0.182 ± 0.006 e \(\text{nm}^{-2}\) and a surface potential of -27.6 ± 1.3 mV. These estimates were then used to describe the relationship between ΔV\(_{0.5}\) and concentration for all three polyamines as shown by the broken curves for polyamines with valences of 4\(^+\), 3\(^+\) and 2\(^+\) (Fig. 3D). The measured ΔV\(_{0.5}\) for spermine and putrescine was less than that predicted by Gouy-Chapman theory. Consequently we expanded the model to include direct binding by polyamines to the surface charge. \(^{33,34}\) The ΔV\(_{0.5}\) for spermine was also well described by inclusion of a binding site with an association constant (K\(_{a}\)) of 10 M\(^{-1}\) and this model estimated values of 0.120 ± 0.005 e \(\text{nm}^{-2}\) and -19 mV for surface charge and potential respectively (Fig. 3D; upper unbroken curve). Inclusion of the binding site model with these values of surface charge and potential better described the concentration-dependence of ΔV\(_{0.5}\) for putrescine and spermidine when combined with K\(_{a}\) of 1 and 3 M\(^{-1}\) respectively (Fig. 3D; middle and lower unbroken curves).

Secondly, the maximum permeability (P\(_{\text{max}}\)) decreased with increasing polyamine concentration (Fig. 3A–C) reflecting a decrease in maximal current flow due to possible channel block. Normalization of P\(_{\text{max}}\) at each concentration of polyamine (P\(_{\text{norm}}\)) facilitated comparison of the action of these molecules on maximal VACC permeability (Fig. 3E). Over the same concentration range there was a modest trend towards an increase in slope factor with polyamine concentration that was of similar amplitude for all three agents (Fig. 3E). Taken together these data are consistent with two mechanisms of inhibition of VACCs by polyamines: a depolarizing ΔV\(_{0.5}\) and a decrease in VACC permeability.

Decreased pH reduces polyamine-mediated VACC inhibition. As polyamines are highly charged we tested whether physiological falls in pH (-0.4 units) that accompany exocytosis\(^{38,39}\) modulated the action of spermidine on VACC currents. We postulated that the
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Polyamines facilitate VACC at increased pH. Polyamines have pKs above 8 and thus increase the pH of physiological solutions on hydration. At high pH less protonated forms of polyamines exist which we postulated might interact differently with VACC than the higher valence forms. The pH of Tyrode solution increased with spermine concentration despite buffering by 10 mM HEPES (Fig. 5A). Application of spermine (3 mM at pH 9.6) shifted the VACC current in a hyperpolarizing direction and increased the current amplitude in the seven cells tested; both actions were possibly attributable to the increase in pH (Fig. 5B). However, at ~20 mV there was an inflection point during the activation phase of the inward current (arrow Fig. 5B) which we had not observed in previous recordings. To examine this unexpected current we applied Cd²⁺ (200 μM) to block the VACC, leaving an inward current that peaked at ~15 mV and then decayed, with no clear reversal potential under these conditions (Fig. 5C). The I-V relationship was consistent with a voltage-activated Na⁺ channel (VANC) current but this seemed unlikely as the recording was made in the presence of

polyamine-induced ΔV₀.₅ would sum with the ΔV₀.₅ observed when pH is decreased, and that this may enhance polyamine-mediated VACC inhibition. The pH-mediated ΔV₀.₅ is well described by models incorporating charge screening and H⁺ binding to at least one site. We examined the actions of spermine and spermidine (5 mM) on SCG VACC currents in normal and lower pH. As expected, reduction of pH shifted the I-V curve to the right and decreased the peak inward current (Fig. 4A). On the P-V plot of these currents, spermine shifted V₀.₅ by 9.6 ± 0.1 mV (n = 2) whereas pH 6.96 shifted the curve by 3.1 ± 0.1 mV (Fig. 4B and C). Application of spermine at pH 6.96 only shifted V₀.₅ by 11.0 ± 1.1 mV. This effect was well described (Fig. 4C, open circles) by incorporation of H⁺ binding and screening with the spermine screening model using a H⁺ association constant of 7.7 x 10⁵ M⁻¹, as reported previously for N-type VACC, and surface charge and potential of 0.245±0.006 e⁻ nm⁻² and -33.2 ± 0.6 mV respectively. However, this model did not explain the action of spermidine at pH 6.96 (Fig. 4D; open circles). As expected from the dose-response data, spermidine shifted V₀.₅ less than spermine 4.9 ± 0.5 mV (Fig. 4D; n = 5, p = 0.005) and pH 6.96 affected ΔV₀.₅ similarly (4.4 ± 0.9 mV; p > 0.05). As with spermine, the P-V relationship was not shifted further (5.2 ± 0.3 mV; p > 0.05) by the co-application of spermidine and low pH (Fig. 4D). In contrast, the model predicted ΔV₀.₅ would increase with the combination of spermidine and pH 6.96 (open circles). Incorporation of a weak binding site for polyamines, using the association constants derived in Figure 3, slightly better described the decreased effects of polyamines when combined with lower pH (Fig. 4C and D, closed circles). The surface charge and potential estimated with this approach were 0.186 ± 0.022 e⁻ nm⁻² and -26.6 ± 2.6 mV, respectively.

**Figure 2.** Polyamines cause dose-dependent inhibition of VACC. VACC currents activated by ramp depolarizations in three exemplar neurons were inhibited by (A) spermine, (B) spermidine, and (C) putrescine. (D) Diary plot of peak inward VACC current versus time and concentration of spermine. (E) Concentration-effect relationship for polyamines on peak VACC currents normalized against current in absence of polyamine. The curves were drawn using equation 1 employing the mean values obtained from fitting the data for each recording. The slope factors H were 0.71 ± 0.06 (n = 10), 0.9 ± 0.03 (n = 5), and 0.77 ± 0.31 (n = 3) for spermine, spermidine and putrescine respectively. The IC₅₀ and H values for putrescine were obtained with proportionately less inhibition and these estimates are more approximate than the measures made using spermine and spermidine.
a saturating concentration (1 μM) of the VANC blocker TTX. To explore this current further and to examine the impact of higher pH on polyamine modulation of VACC currents, we performed conventional voltage-step experiments at lower concentrations of TTX (0.2 μM).

The inward current measured at the end of the 20 ms step (I_{late}) was identified as a VACC current based on the recording solution configuration and its sensitivity to Cd^{2+} (10 mM Ca in bath solution, Fig. 5D) at high and physiological pH. Spermidine (5 mM) reduced the VACC current amplitude at pH 7.35 (Fig. 5E). The higher pH had little effect on the VACC current at 0 mV in the absence of spermidine but co-application of spermidine at pH 10.35 modestly increased the late Cd^{2+}-sensitive current (Fig. 5D, E and G). At pH 10.35 the I-V relationship was shifted in a hyperpolarizing direction (Fig. 5G) by over 10 mV, which was considerably more than the -2.4 mV predicted by the model employing H^+ screening and H^+ binding (K_s = 7.7x10^5 M^{-1}). In contrast, application of spermidine at pH 10.35 did not depolarize ΔV_{0.5}. The majority of spermidine

Figure 3. Polyamines inhibit VACC by causing a depolarizing shift in channel gating and by decreasing steady state VACC permeability. [A–C] The permeability vs voltage plots were derived by transforming currents in Figure 2 A–C using GHK equation. [D] The mean (± SE) shift in V_{0.5} (ΔV_{0.5}) relative to V_{0.5} in the absence of polyamine is plotted against spermine (○; n = 10), spermidine (■; n = 5), and putrescine (▲; n = 3) concentration. Broken curves show plots of Grahame equation to the polyamine data based on a surface charge of 0.182 ± 0.006 e·nm² and a surface potential of -27.6 ± 1.3 mV. The unbroken curves show fits to model incorporating surface charge of 0.120 e·nm², surface potential of -19 mV and binding site with K_s of 10, 3, and 1 M^{-1} for spermine, spermidine and putrescine respectively. [E] The mean steady state P normalized against P in the absence of polyamines versus polyamine concentration. [F] The mean slope factor versus polyamine concentration. Symbols as for (D).
much larger early, rapidly inactivating current was observed (Fig. 5E) and its amplitude increased substantially with co-application of spermidine. In support of the hypothesis that the early and late currents were carried by distinct channels, they developed with different rates following application of high pH and spermidine (Fig. 5F). The I-V plot of the early current showed a peak at -20 mV at pH 10.35 and substantially larger early currents in the presence of spermidine (Fig. 5H). In the absence of TTX a larger early current was observed (data not shown). Both of these currents reversed at the same potential (E_r) of -40 mV. The similar E_r and rapid activation and inactivation are consistent with the early current being carried by VANCs. Furthermore the early inward current recorded at pH 10.35 in the presence of spermidine and TTX (0.2 µM) was blocked by increasing TTX to 2 µM, and the early current E_r shifted by between -15 and -20 mV when extracellular [Na+] was decreased from 150 to 75 mM. TTX still blocked VANC currents following retitration to pH 7.35 after 2 hours at pH 10 (n = 4), indicating the loss of effect was not due to irreversible toxin destruction. These data support the hypothesis that at high pH the addition of polyamines reduces the potency of TTX to block VANCs. This may arise if polyamines and elevated pH decrease the association rate or increase the dissociation rate of TTX for VANC.

We examined the block by TTX at pH 7.35 and in the presence of 3 mM spermine at pH 9.6. The exemplar traces confirm that the VANC current block by TTX (200 nM) at steady state is reduced by spermine (pH 9.6) from 96 ± 0.4% to 69 ± 3% (Fig. 6A). The normalized diary plots of time course of action show exponential decreases in the peak VANC current amplitudes with the application at time zero of TTX or TTX plus spermine (Fig. 6B). The average time constants for the curves fit to the diary plots slowed from 6.0 ± 0.9 s (n = 9) to 20.6 ± 4.9 s (n = 3) at 200 nM when spermine was co-applied with TTX (p = 0.009). In addition to slowing the on-rate of TTX, spermine appeared to hasten the TTX off-rate (Fig. 6C).

The peak VANC currents increased exponentially when the neurons were perfused with TTX free solution at pH 7.35. Fitting the two exemplar diary plots gave time constants of 99 and 83 s for TTX and TTX plus spermine and a trend towards a decrease for the average data (124 ± 22 s (n = 4) and 78 ± 3 s (n = 3); p = 0.057).

One possibility not excluded by the above findings is that polyamines directly activate VANC currents. We tested this hypothesis by comparing the action of high pH and spermidine VANC currents in the absence of TTX in two neurons. The currents activated by depolarizing steps from -70 mV to voltages between -40 and -10 mV were unaffected by spermidine at pH 7.35 (Fig. 7A). Application of bathing solution at pH 10.35 lead to a clear increment in the current amplitudes following steps to -30 and -20 mV. There was no additional increase in these currents when spermidine was applied at pH 10.35 in contrast to when spermidine was applied with TTX (Figs. 5E and 7A). This finding is also illustrated by the I-V plots (Figs. 5H and 7B). While the I-V curves were shifted in a hyperpolarizing direction by high pH, spermidine did not produce any additional changes (Fig. 7) indicating that these VANCs are not activated directly by spermidine.

**DISCUSSION**

Polyamines have been shown to modulate a variety of ion channels, both intracellularly and extracellularly.\(^1\)\(^5\) We confirm here...
Figure 5. Elevated pH and polyamines hyperpolarize activation of VACC and increase VANC currents in the presence of TTX. (A) Spermine increases pH in HEPES-buffered Tyrodes solution. (B) VACC currents are increased and left shifted by co-application of pH 9.6 and 3 mM spermine (arrow indicates inflection point and solution configuration). [C] Inward current responsible for inflection point revealed by application of Cd2+ (100 µM) and TTX (1 µM). (D) Differential TTX and Cd2+ sensitivity allow pharmacological separation of Iearly and Ilate activated by steps to 0 mV from -70 mV. (E) Action of high pH and spermidine (5 mM) on current traces activated by step voltage from -70 to 0 mV in a different neuron (0.2 µM TTX and Ca2+/Mg2+:1.1 mM/1.1 mM). (F) Diary plot of Iearly (□) and Ilate (■) following switch from pH 7.35 to pH 10.35 + spermidine. The curves are both fit well by single exponential curves with time constants of 48 and 26 s for Iearly and Ilate respectively. (G) The I-V relationship for Ilate at pH 7.35 and 10.35 in the presence and absence of 5 mM spermidine. The current recorded at -40 mV with pH 10.35 plus spermidine was omitted as it is contaminated by a large, slowly inactivating Iearly. (H) The I-V relationship for Iearly at pH 7.35 and pH 10.35 in the presence and absence of 5 mM spermidine. (E-H) were recorded from the same neuron.
earlier reports that extracellular polyamines inhibit neuronal VACC currents but report a number of new findings. First, polyamines inhibit VACC currents in SCG neurons by shifting $V_{0.5}$ to depolarizing potentials and by reducing $P_{\text{max}}$. Second, the inhibition of VACC is greatest in the order spermine > spermidine > putrescine. Third, acidification of extracellular solution shifts the $V_{0.5}$ but this effect does not sum with the similar shift produced by spermidine. Fourth, many of the polyamine actions on VACC are predicted by the Gouy-Chapman-Stern theory. Finally, spermidine reduces the potency of TTX at VANCs at elevated pH but does not directly activate sodium channels.

**Polyamines and surface charge screening.** We report here that polyamines modulate VACC and VANC by a number of different mechanisms. We determined that the VACC current inhibition was due to both a depolarizing $\Delta V_{0.5}$ and a decrease in $P_{\text{max}}$ (Fig. 3D and E) although the former effect was absent with putrescine. The effects on $\Delta V_{0.5}$ for spermine were attributable to surface charge screening by the tetravalent cation, using Gouy-Chapman theory. The trivalent and divalent spermidine and putrescine produced less $\Delta V_{0.5}$ than predicted by the model, and fits were improved by the addition of binding to a low affinity site ($K_s = 10, 3$ and $1 \text{M}^{-1}$ for spermine, spermidine and putrescine respectively). Like spermine and spermidine, low pH shifted the activation of VACC currents in a depolarizing direction (Fig. 4). The pH induced shift and size change in inward current has been reported for other VACC, and been shown to reflect voltage shift of channel activation as a result of the titration of binding sites for surface charge. The decreased shift in $\Delta V_{0.5}$ when $H^+$ was co-applied with spermine or spermidine was partly explained by the inclusion of $H^+$ binding (Fig. 3E) this did not seem warranted. The surface charge of 0.120 ± 0.005 e nm$^{-2}$, estimated from the spermine experiments assuming a binding site ($K_s = 10 \text{ M}^{-1}$), was lower than reports for mammalian P/Q type (0.23 e nm$^{-2}$) and bullfrog N-type VACC (0.42 e nm$^{-2}$ when $K_s = 10 \text{ M}^{-1}$). However experiments incorporating $H^+$ binding ($K_s = 7.7 \times 10^3 \text{ M}^{-1}$) yielded a value closer to these earlier reports (0.186 ± 0.022 e nm$^{-2}$).

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Polyamine Modulation of VACC

We also demonstrated that the VACC currents were increased and $\Delta V_{0.5}$ hyperpolarized by high pH as observed by others. In contrast to experiments at pH 7.35 the co-application of spermine and spermidine did not depolarize $\Delta V_{0.5}$. At the higher pH, spermidine exists in three forms (see above) and is mainly monovalent rather than the $3^+$ at pH 7.35. Following incorporation of the concentrations and valences for the three spermidine species, the model predicted that 5 mM spermidine shifted $\Delta V_{0.5}$ by 0.4 mV at pH 10.35 but by 5.4 mV at pH 7.35. This approach went some way to explaining why spermidine did not cause a significant depolarization of $V_{0.5}$ at high pH however there were important limitations to this model including the underestimation of the 10–20 mV hyperpolarizing $\Delta V_{0.5}$ for VACC at pH 10.35. The further hyperpolarizing shift of the I-V curve following application of spermidine and pH10.35 was also not predicted by the model. Other work is needed to determine if the fits can be improved by including additional binding sites for H$^+$ as suggested by others. These discrepancies may also reflect limitations in the assumptions of the model such as the uniformity of the distribution of membrane surface charge, that the polyamines can be considered point charges, and the absence of interactions between permeating ions and polyamines.

Alterations in charge screening have also been shown to weakly impact VACC permeation as well as gating. However this seems an unlikely explanation for the observed concentration-dependent decreases in $P_{\text{max}}$ since all three polyamines produced similar changes (Fig. 3E) but had such different effects on $\Delta V_{0.5}$. Another explanation is that the polyamines also blocked the VACC. The lack of inhibition of the outward currents (Fig. 2A–C) raises the possibility that such block might be voltage-dependent. A modest increase in k with polyamine concentration (Fig. 3F) indicates that polyamines are also decreasing the sensitivity of VACC activation to depolarizing voltages presumably by binding to the channel.

Overall the data within the normal to modestly reduced pH range is reasonably well described by a model incorporating surface charge screening, binding sites for H$^+$ and polyamines, and channel block.

Physiological actions of polyamines. Interest in possible extracellular actions of polyamines in the nervous system arose with the proposals that these intracellular polyvalent may be endogenous signaling molecules, and that they can modulate synaptic plasticity. The N-type VACC was proposed as a target for polyamines due to the interaction observed in binding and functional studies. Previous measurements of polyamine actions have suggested that the affinity of spermine for VACC approached 10 mM but these experiments were conducted using higher extracellular divalent concentrations in sensory neurons, which contain a mixture of types of VACC types. Both of these factors could have lead to overestimates of the IC$_{50}$. We report that the IC$_{50}$ for spermine is 4.7 mM. Electrophysiological studies have shown that spermine and spermidine reduced excitatory transmission in the hippocampus and increased paired-pulse facilitation at submillimolar concentrations, both findings consistent with inhibition of a presynaptic VACC.

Our measurements of the affinity of polyamines for VACCs strongly suggest that inhibition of N-type VACC does not underlie spermine- and spermidine-mediated neuroprotection or inhibition of synaptic transmission as has been suggested. Furthermore the relatively high IC$_{50}$ of spermine for VACC compared with the ~3 mM concentration estimated in synaptic vesicles suggests that other targets with lower IC$_{50}$, such as the polyamine sensitive [Ca$^{2+}$]-sensing on cortical nerve terminals (~7 μM for spermine- Chen and Smith, unpublished observations), are more likely to mediate physiological actions of endogenously released polyamines. We also addressed whether the affinity of polyamines was increased by physiological changes in external pH but our data showed that falls and rises in pH attenuated polyamine-mediated inhibition of VACC (Figs. 4 and 5). Taken together our data argue strongly against the inhibition of VACC by polyamines underlying their actions on synaptic transmission or neuroprotection. However such effects cannot be completely discounted since inhibitory effects were demonstrated in the hippocampus with concentrations of 100 μM, and modest inhibitory effects on VACC at this dose (Fig. 2) may have significant effects on synaptic transmission especially at lower extracellular [Ca$^{2+}$].

Polyamines at elevated pH reduce TTX block of VANC. Elevating extracellular pH left-shifted and increased the maximal amplitude of the I-V plot of VANC currents recorded from SCG neurons (Fig. 7). The -10 mV shift we observed, which has been attributed to titration of negative fixed charges on external membranes, was slightly greater than reported for VANC in peripheral nerve and myocardium and is consistent with a lower affinity for the H$^+$ binding site in SCG neuron VANC isoforms. This change would make the SCG VANC more sensitive to elevated pH but probably still unlikely to mediate significant changes in neuronal excitability during the modest physiological increases in extracellular pH.

Polyamines decreased the association and tended to increase the dissociation rates of TTX at higher pH leading to activation of VANC currents in the presence of TTX (Figs. 5 and 6). The loss of potency of TTX at higher pH was reversed by reduction of pH and has been previously attributed to reduced effectiveness of the zwitterion form of TTX following decreased protonation. In addition we observed a polyamine-mediated inhibition of TTX which may have been due to an action on the VANC or an action on TTX. Since VANC currents were relatively unaffected by spermidine (Fig. 7) it seems more likely that spermidine and spermine were reducing TTX action at elevated pH by interacting with TTX and producing a structural change. Another explanation is that at increased pH the decreased polyamine valence and subsequent increased surface charge would lead to decreased local TTX concentration in the vicinity of the VANC, and so reduce the action of TTX.

In conclusion, polyamines inhibit neuronal VACCs via complex interactions with extracellular H$^+$ and Ca. Many of the observed effects can be explained by a model incorporating polyamine binding, H$^+$ binding and surface charge screening.

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