

Spermidine Release from *Xenopus* Oocytes

ELECTRODIFFUSION THROUGH A MEMBRANE CHANNEL*

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Qun Sha, Carl Romano‡, Anatoli N. Lopatin, and Colin G. Nichols§

From the Departments of Cell Biology and Physiology, and ‡Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

The mechanism of spermidine release from *Xenopus* oocytes was examined by measuring release of radioactive [³H]spermidine under different ionic conditions, and under voltage-clamp. In normal solution (2 mM K⁺), the efflux rate is less than 1% per hour, and is stimulated ~2-fold by inclusion of Ca²⁺ (1 mM) in the incubation medium. Spermidine efflux is stimulated ~10-fold in high [K⁺] (KD98) solution. In KD98 solution, efflux is strongly inhibited by divalent cations (K_i for Ba²⁺ block of spermidine efflux is ~0.1 mM), but not by tetraethylammonium ions or verapamil. Spermidine efflux rates were not different between control oocytes and those expressing HRK1 inward rectifier K⁺ (Kir) channels. When the membrane potential was clamped, either by changing external [K⁺] in oocytes expressing HRK1, or by 2-microelectrode voltage-clamp, spermidine efflux was shown to be strongly dependent on voltage, as expected for a simple electrodiffusive process, where spermidine³⁺ is the effluxing species. This result argues against spermidine diffusing out as an uncharged species, or in exchange for similarly charged counterions. These results are the first conclusive demonstration of a simple electrodiffusive pathway for spermidine efflux from cells.

Polyamines (spermine, spermidine, and putrescine) are present in almost all cells (Tabor and Tabor, 1984), and have important roles in stabilizing DNA. Recently, they have been implicated in physiological regulation of potassium channels, from inside cells (Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Fakler *et al.*, 1995), and of glutamate receptor channels from outside (Usherwood and Blagborough, 1994; Romano and Williams, 1994). Polyamines are taken up by, and released from, cells, but the mechanisms remain poorly understood (Khan *et al.*, 1994). In general, polyamine *influx* is reported to be saturated at low micromolar concentrations (Seiler and Dezeure, 1990; Khan *et al.*, 1991). Polyamine *efflux* has received less attention, although there is a general consensus that both influx and efflux depend on the membrane potential (Khan *et al.*, 1994; Shaw, 1994). Polyamine transporters have been cloned from bacteria (Furuchi *et al.*, 1991; Kashiwagi *et al.*, 1993), but no evidence is available on the molecular nature of eukaryotic

transport systems.

We recently reported that polyamines cause inward rectification of potassium (Kir) channels (Lopatin *et al.*, 1994; Ficker *et al.*, 1994; Fakler *et al.*, 1995), by steeply voltage-dependent block of the channel pore. Kir channels are present in most cells, and polyamine-induced rectification is likely to be ubiquitous. Detailed biophysical examination of this process suggests that in blocking the Kir channel, polyamines traverse a significant fraction of the membrane electric field (Lopatin *et al.*, 1995). It is possible that polyamines might actually permeate the channel fully, but behave macroscopically as channel blockers due to a long residence time within the pore. Accordingly, we were prompted to examine the possibility that efflux through Kir channels is a significant pathway for spermidine efflux. The results suggest that this is not the case, but provide significant information on the mechanism by which spermidine efflux does occur. First, under voltage-clamp conditions, spermidine efflux is strictly dependent on membrane potential, as expected for a trivalent species. Second, voltage-dependent spermidine efflux is strongly inhibited by Ba²⁺ and Ca²⁺, but not tetraethylammonium ions or verapamil. We conclude that efflux of trivalent spermidine occurs through an electrodiffusive pathway, and hypothesize that the pathway is through cation channels in the cell membrane. Such a pathway may be a universal route of polyamine flux in different cells.

MATERIALS AND METHODS

Oocyte Maintenance and Expression of Kir Channels—cDNAs were propagated in the transcription-competent vector pBluescript SK(–) in *Escherichia coli*. Capped cRNAs were transcribed *in vitro* from linearized cDNAs using T7 RNA polymerase. Stage V-VI *Xenopus* oocytes were isolated by partial ovariectomy under tricaine anesthesia, and defolliculated by treatment with 1 mg/ml collagenase (Sigma Type 1A; Sigma) in zero Ca²⁺ ND96 (below) for 1 h. Two to 24 h after defolliculation, oocytes were pressure-injected with ~50 nl of 1–100 ng/μl cRNA. Oocytes were kept in ND96 + 1.8 mM Ca²⁺ (below), supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) for 1–7 days prior to experimentation.

Electrophysiology—Oocytes were voltage-clamped at room temperature using a commercial voltage-clamp amplifier (Warner Instruments, Inc.) in a small chamber (volume 200 μl) mounted on the stage of a SMZ-1 microscope (Nikon Instruments). Electrodes were filled with 3 M KCl and had tip resistances of 1–5 MΩ. PClamp software and a Labmaster TL125 D/A converter were used to generate voltage pulses. Data were normally filtered at 1 kHz, signals were digitized at 22 kHz (Neurocorder, Neurodata, NY) and stored on video tape. Experiments could then be replayed onto a chart recorder, or digitized into a microcomputer using Axotape software (Axon Instruments). Alternatively, signals were digitized on-line using PClamp, and stored on disk for off-line analysis.

Solutions—ND96 solution contained (mM): NaCl 96, KCl 2, MgCl₂ 1, HEPES 5, pH 7.5. KD98 solution contained (mM): KCl 98, MgCl₂ 1, HEPES 5, pH 7.5. Additions to these solutions are described in the text.

Release of [³H]Spermidine from Oocytes Expressing HRK1 Channels—Oocytes were injected with ~50 nl of 70 μM [³H]spermidine (specific activity = 15 Ci/mmol) in 10 mM unlabeled spermidine. Oocytes were incubated for 1–2 h in ND96, or overnight, before experimen-

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§ To whom all correspondence and reprint requests should be addressed. Tel.: 314-362-6630; Fax: 314-362-7463; E-mail: cnichols@cellbio.wustl.edu.

tion. After two washes, first in ND96, then in experimental solution, oocytes were incubated in 0.5 ml of experimental solution. The experimental solution was removed, the oocyte washed in an additional 0.5 ml of experimental solution which was pooled with the first. If a second experimental solution was to be applied, the oocyte was washed in 0.5 ml of this solution, then incubated and washed as above. After all test solutions had been collected, oocytes were lysed overnight in 0.5 ml of 1% sodium dodecyl sulfate (in ND96), before counting. Radioactivity was measured in a scintillation spectrometer.

In voltage-clamp experiments, oocytes were incubated in 200 μ l of solution. At the end of the test period, the solution in the chamber and 2 additional volumes of wash solution were collected, without disturbing the oocyte or the electrodes. Oocytes were washed with a further 3 volumes of the next test solution, before beginning the next test period.

HPLC¹ Analysis of Released Polyamines—HPLC analysis was performed to determine the chemical nature of the released radioactivity (modified from Minocha *et al.* (1990) and Smith and Davies (1985)). Pooled media containing released counts were alkalized with one-tenth volume of saturated sodium carbonate and then passed over a SepPak cartridge (Millipore) to adsorb polyamines and potential metabolites. After washing the cartridge with water, the concentrated and partially purified amines were eluted with 1 ml of trifluoroacetic acid (0.1%). A mixture of unlabeled polyamines (1 nmol each of putrescine, cadaverine, spermidine, and spermine) was added to serve as carrier and internal standard. The spiked, acidic samples were alkalized with saturated Na_2CO_3 . Dansyl chloride (0.5 ml of 1 mg/ml in acetone) was added and the reaction proceeded for 20 min at 65 °C in the dark. The reaction mixtures were removed from the 65 °C bath and proline (100 μ l of 250 mg/ml) was added to quench unreacted dansyl chloride. After 30 min, derivatized amines were adsorbed onto small C18 columns (ExtractClean, Alltech) equilibrated with 35% acetonitrile, washed with 2 ml of 35% acetonitrile, and eluted under vacuum with 100% acetonitrile. The eluate was concentrated under vacuum, centrifuged, and aliquots were chromatographed on a C18 reverse-phase column (Rainin), and eluted via an acetonitrile gradient. The column effluent was collected in scintillation vials and counted. Dansylated polyamines were detected using an in-line fluorescence spectrophotometer (Model FL-750-BX, McPherson, Acton, MA). Output was recorded on a Macintosh computer equipped with an A-D converter and data acquisition/analysis software (Rainin).

RESULTS

Spermidine Efflux from *Xenopus* Oocytes, Dependence on Ionic Conditions—Fig. 1 illustrates the time dependence of [^3H]spermidine efflux from control *Xenopus* oocytes, and from oocytes expressing Kir (HRK1) channels (Makhina *et al.*, 1994), in normal 2 mM [K^+] solution (ND96). In ND96 solution, the efflux rate is approximately 1% per hour, irrespective of the expression of HRK1 channels, and is stimulated ~2-fold in the presence of Ca^{2+} (1 mM). Efflux is strongly stimulated by incubation in high [K^+] (KD98) solution (Fig. 1B). In order to verify that the radioactivity released from [^3H]spermidine injected oocytes is still incorporated into spermidine, we performed HPLC analysis of dansylated fractions of released counts. All radioactivity above background is present in spermidine (Fig. 1C), suggesting that there is minimal metabolism of the injected [^3H]spermidine to other species up to 24–27 h after injection.

High [K^+] Induced Spermidine Release Is Inhibited by External Divalent Cations—Although Ca^{2+} ions stimulate release in ND96 solution (Figs. 1 and 2C), spermidine release is significantly inhibited by Ca^{2+} in KD98 solution (Fig. 2C). Thus there is a 2-fold action of Ca^{2+} on spermidine release; Ca^{2+} inhibits K^+ -induced release, but stimulates K^+ -independent release. The remainder of this paper examines specifically the K^+ -induced release. Extracellular Ba^{2+} also strongly inhibits spermidine efflux in high [K^+] from both control and HRK1-expressing oocytes (Fig. 2A), with $K_i \sim 0.1$ mM (Fig. 2B). The results in Figs. 1 and 2 confirm that significant spermidine

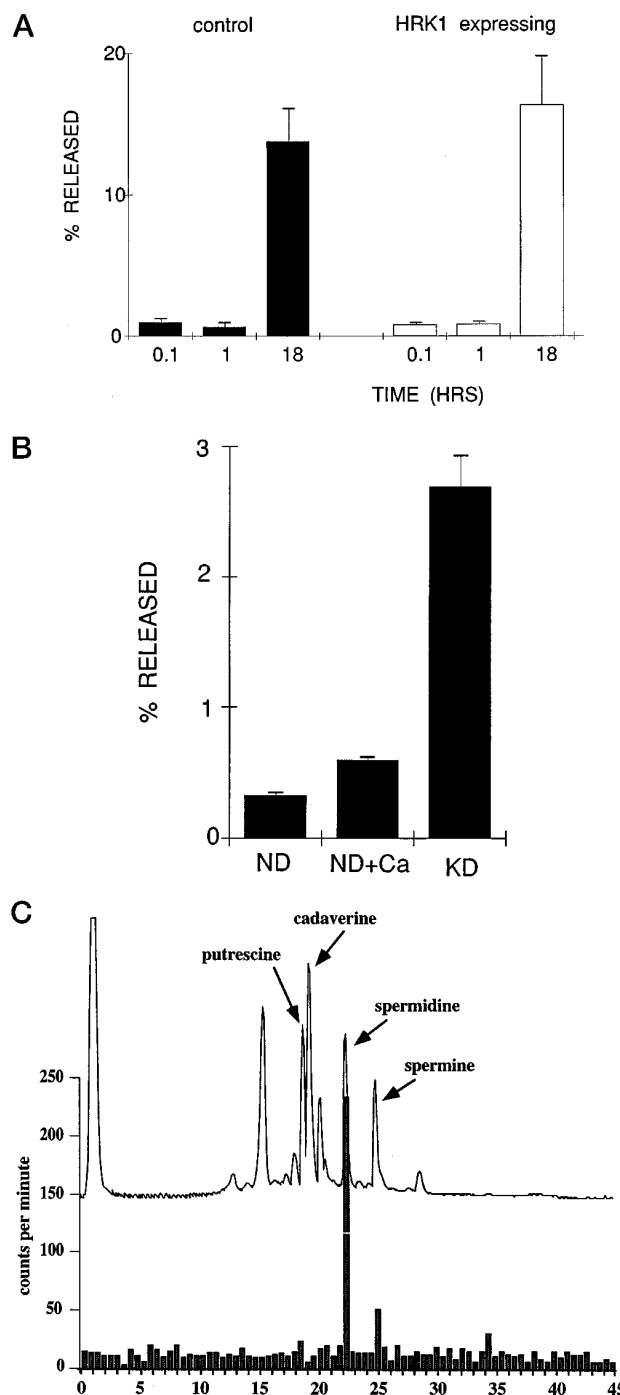


FIG. 1. [^3H]Spermidine release from *Xenopus* oocytes. A, [^3H]spermidine released into the medium (as a percentage of the total injected into the oocyte) in 0.1, 1, and 18 h, for control oocytes, and oocytes expressing HRK1 inward rectifier K^+ channels. These experiments were performed in ND96 solution without Ca^{2+} . The error bars show mean \pm S.E. in this and other figures ($n = 12$ oocytes in each case). B, [^3H]spermidine released in 90 min from HRK1 expressing oocytes incubated in ND96 (ND), ND96 + 1 mM Ca^{2+} , or KD solutions. C, elution profile of different polyamine species (fluorescence), and of tritiated species released from oocytes during a 3-h incubation in KD98 solution. Oocytes were injected 24 h previously. The same pattern of radioactivity was observed for oocytes injected 1 h prior to release assay.

efflux does not occur through HRK1 channels, but do not discount the possibility of enhanced efflux at strong depolarizing potentials, where the entry of polyamines into the Kir channel is favored (Lopatin *et al.*, 1994).

Using [K^+] to Control Membrane Potential Reveals Voltage-

¹ The abbreviations used are: HPLC, high performance liquid chromatography; TEA, triethanolamine.

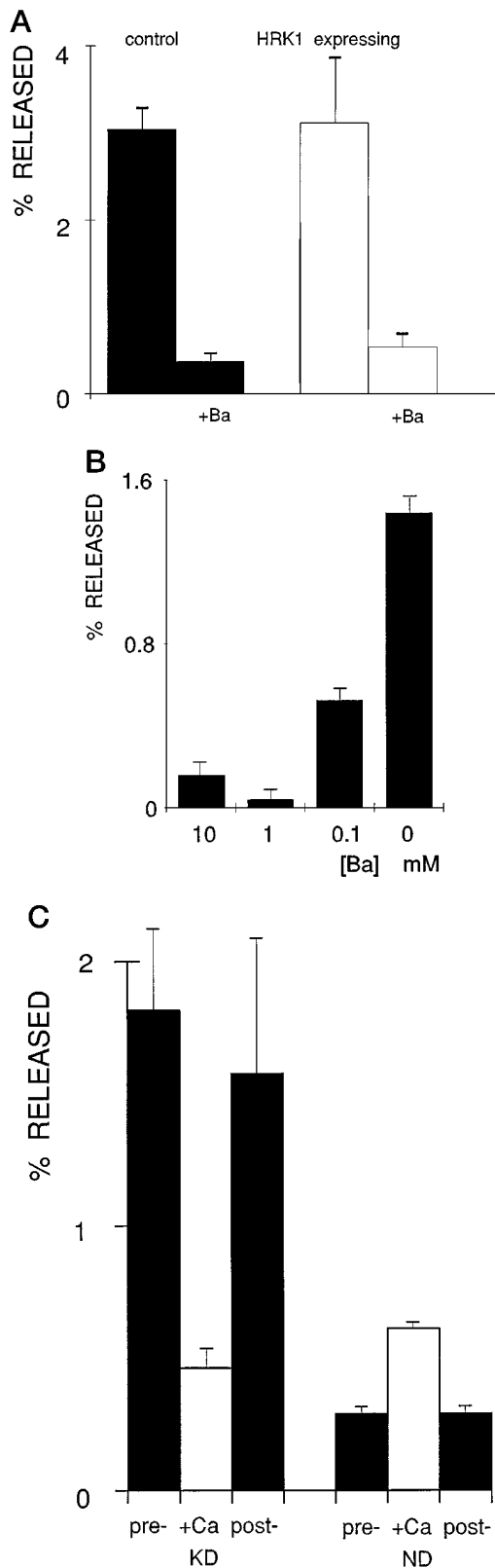


FIG. 2. Divalent cations inhibit K⁺ stimulated [³H]spermidine release. A, [³H]spermidine released in 90 min from control and HRK1 expressing oocytes incubated in KD98 solution \pm 1 mM Ba²⁺. Graph shows mean \pm S.E. for $n = 7$ oocytes in each case. B, [³H]spermidine released in 90 min from HRK1 expressing oocytes incubated in KD98 solution plus 0–1 mM Ba²⁺. Graph shows mean \pm S.E. for $n = 4$ –18 oocytes. C, [³H]spermidine released in 90 min from HRK1 expressing oocytes incubated in KD98 (KD), or ND96 (ND), solutions in the absence of Ca²⁺ (pre-), after inclusion of 1 mM Ca²⁺ in the medium (+Ca), and after removal (post-) of Ca²⁺ from the incubation medium ($n = 6$ oocytes in each case).

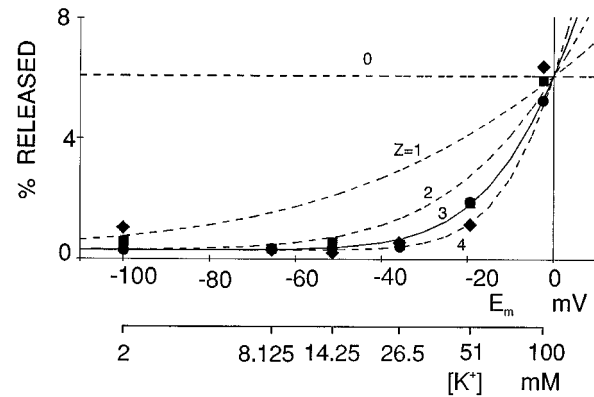


FIG. 3. In the absence of divalent cations, [³H]spermidine release is by electrodiffusion. [³H]Spermidine released in 120 min from HRK1 expressing oocytes versus E_m . E_m was calculated from the potassium reversal potential (E_K). E_K was varied by changing [K⁺] in the incubation medium by mixing KD98 and ND96 solutions. Results are shown for 3 oocytes for which all solution changes were successfully completed. Solutions changes were made in increasing concentration of potassium (■), or in decreasing concentration of potassium (●, ◆). The dashed lines indicate the relationship predicted for simple electrodiffusion of a species with 0, 1, 2, 3, or 4 charges according to the GHK equation (see text).

dependence of Spermidine Efflux—The above results demonstrate that in the absence of external divalent ions, spermidine efflux is stimulated by raising external [K⁺], consistent with a role of membrane potential (E_m). This finding was examined more rigorously by measuring external [K⁺]-dependence of spermidine release in oocytes expressing HRK1 channels. In control oocytes, E_m may be unpredictable due to variability of Cl[−] and K⁺ conductances. High density expression of K⁺-selective HRK1 channels increases the membrane conductance >100-fold (Makhina *et al.*, 1994), ensuring that E_m will faithfully follow the potassium reversal potential (E_K) as external [K⁺] (K_o) is altered. Fig. 3 shows spermidine efflux, plotted as a function of K_o or of E_m . The dashed lines in Fig. 3 show the voltage-dependence of efflux that is predicted by simple electrodiffusion (Eq. 1) for uncharged, mono-, di-, tri-, or tetravalent species, according to the Goldman-Hodgkin-Katz “current” equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$\text{Flux} = Pz^2(E_m F^2/RT) \cdot (X_i - X_o \exp(-zFE_m/RT)) / (1 - \exp(-zFE_m/RT)) \quad (\text{Eq. 1})$$

where p = permeability, z = charge of the permeating ion, X_i and X_o are the internal and external concentrations of the permeating ion (in this case $X_o = 0$), E_m is the membrane potential, and F , R , and T have their usual meanings.

Two-microelectrode Voltage-clamp Experiments Provide Direct Evidence for Voltage-dependent Spermidine Efflux—It is apparent that spermidine efflux is well described as a simple electrodiffusive process, where spermidine³⁺ is the effluxing species. This result argues against spermidine diffusing out as an uncharged species, or in exchange for similarly charged counterions, since the voltage-dependence of efflux would not then be as steep. It is plausible that the relevant parameter determining spermidine efflux, is not actually voltage, but [K⁺], or [Na⁺], if for instance, spermidine efflux occurred through a K⁺/spermidine exchanger or a Na⁺/spermidine co-transport. These possibilities are excluded by the experiments shown in Fig. 4. Spermidine efflux was measured from voltage-clamped oocytes, in both high [K⁺] (KD98) and low [K⁺] (ND96) solutions. Individual oocytes were clamped first to a control voltage of −90, or 0 mV, for 30 min, then to a test voltage for 30 min, then once more to the control voltage. Such a protocol (Fig.

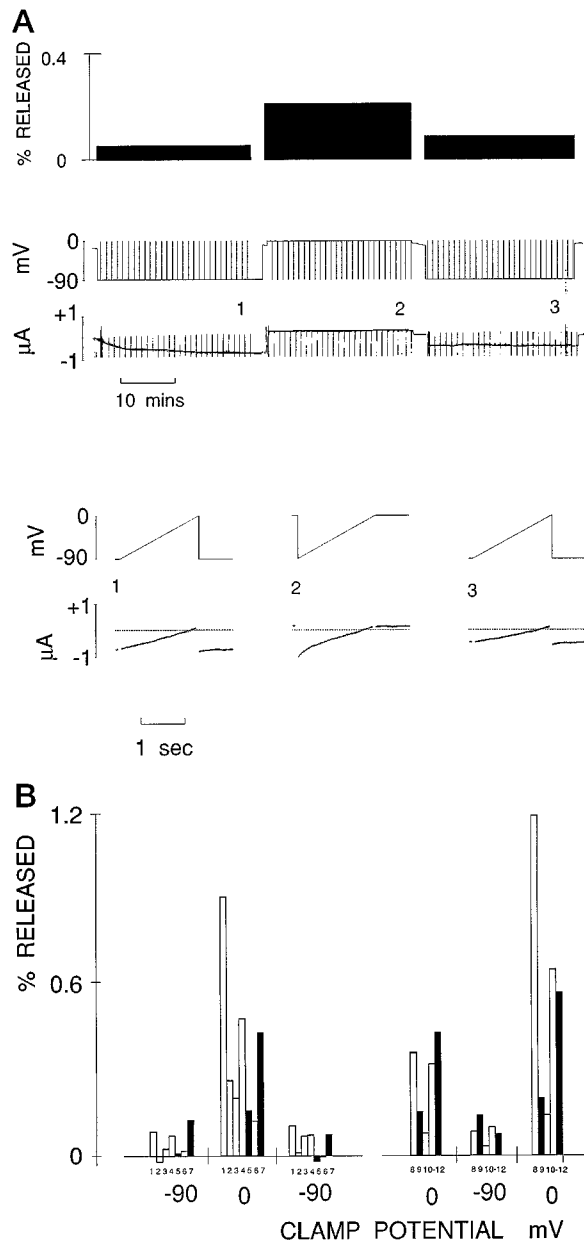


FIG. 4. **[³H]Spermidine release is not dependent on ion composition of the medium.** Upper panel, slow time base record of E_m and membrane current, from an oocyte clamped at a holding potential of -90 mV (left), then 0 mV (middle), then -90 mV (right) for 30 min in each case. The oocyte was bathed in ND96 solution. The top panel shows [³H]spermidine released during each 30-min period. During each period, the E_m was ramped from -90 to 0 mV every minute, and sample fast time base records of E_m and membrane current are shown in the bottom panel, from the times indicated. B, results of individual experiments (numbered) like those in A, for voltage-clamp to -90 , then 0 , then -90 mV (oocytes 1–7), or to 0 , -90 , and 0 mV (oocytes 8–12). Experiments in ND96 are shown as white boxes, experiments in KD98 are shown by black boxes.

4A) ensured that changes in efflux rate were reversible, and not simply due to progressive changes as a result of long duration voltage-clamp experiments (1–2 h). The increased spermidine efflux during voltage-clamp at depolarized holding potentials is not a consequence of any significant increase in cell membrane conductance, as shown by the current traces obtained during voltage ramps (Fig. 4A, lower panel). Fig. 4B shows the results of individual experiments using this protocol, performed in both KD98 and ND96 solutions. As is clear from the averaged data (Fig. 5A), although spermidine efflux is significantly stim-

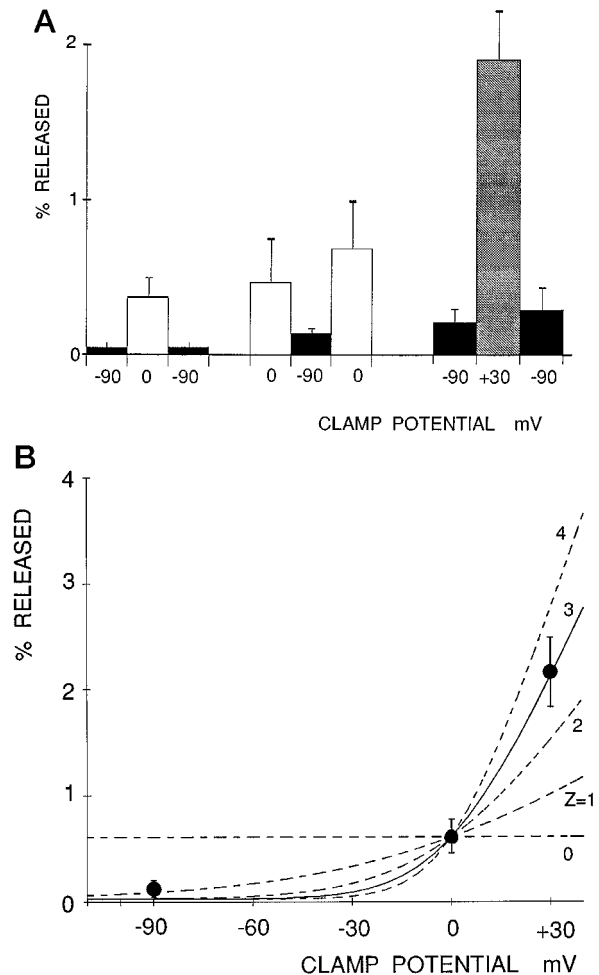


FIG. 5. **Spermidine release under voltage-clamp is by electrodiffusion.** A, averaged results for [³H]spermidine released from voltage-clamped oocytes, clamped to a control voltage (either -90 mV, or 0 mV), then to a test voltage (either 0 , -90 , or $+30$ mV), then once more at the control voltage. Results of experiments performed in ND96 and KD98 solutions are pooled. Graph shows mean \pm S.E. for $n = 7$ (left panel), $n = 6$ (middle panel), and $n = 4$ oocytes (right panel). B, all data from B are pooled and plotted versus voltage-clamp potential. Dashed lines indicate the relationship predicted for simple electrodiffusion of a species with 0, 1, 2, 3, or 4 charges according to the GHK equation (see text).

ulated at depolarized voltages, this is not a result of any significant increase in membrane conductance. Fig. 5A shows averaged data obtained from experiments clamping to -90 , 0 , and $+30$ mV in various sequences. In Fig. 5B, all data obtained at each of three voltages were pooled and plotted on the same graph. The curves are the same as those in Fig. 3, but extended to include the wider voltage range that could be explored in such experiments. It is clear that spermidine efflux does indeed depend on voltage, and that external $[K^+]$ is not a direct determinant of spermidine efflux. These experiments, by permitting voltage-clamp to potentials >0 mV, further extend the data in Fig. 3, and show that spermidine efflux is strongly voltage-dependent and still explainable by electrodiffusion, up to at least $+30$ mV.

Ca^{2+} sensitivity of release is maintained under voltage-clamp conditions (Fig. 6). Thus, depolarization (from -90 to 0 mV) strongly stimulates release in the absence of Ca^{2+} , but there is no stimulation in the presence of 1 mM Ca^{2+} . The background stimulation by Ca^{2+} under hyperpolarized conditions in nonvoltage-clamped oocytes (Fig. 1B) is also apparent under voltage-clamp at -90 mV (Fig. 6).

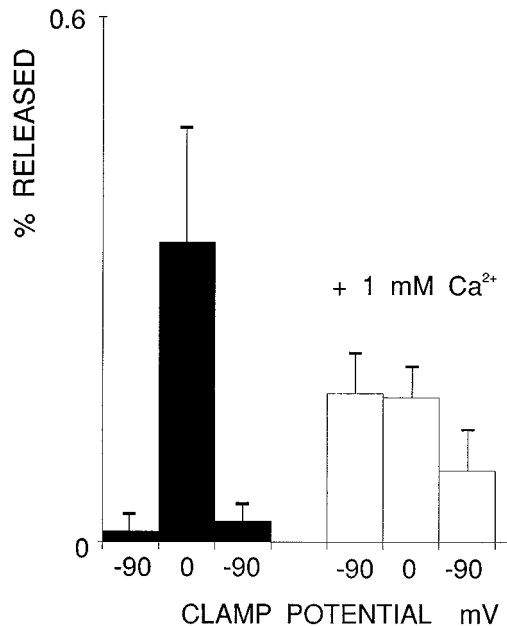


FIG. 6. Calcium sensitivity of release is maintained under voltage-clamp conditions. [^3H]Spermidine released in 30 min from voltage-clamped oocytes incubated in ND96, solution in the absence (left) or presence (right) of 1 mM Ca^{2+} . Oocytes were clamped to -90 mV for 30 min, then to 0 mV for 30 min, then to -90 mV once more for 30 min ($n = 4$ oocytes in each case).

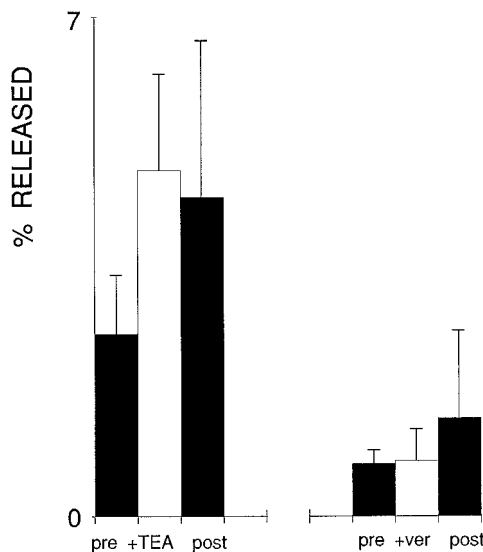


FIG. 7. Spermidine release is insensitive to TEA^+ and verapamil. [^3H]Spermidine released in 90 min (left) or 30 min (right) from HRK1 expressing oocytes in KD98 solution ± 1 mM tetraethylammonium (TEA, left), and ± 10 μM verapamil (ver, right) ($n = 8$ oocytes in each case).

These results show that a major pathway of spermidine efflux is through a simple electrodiffusive pathway that is blockable by Ba^{2+} and Ca^{2+} ions, consistent with efflux through a membrane cation channel. Many cation channels are also blocked by tetraethylammonium (TEA) ions or verapamil (Hille, 1992), but as shown in Fig. 7, neither agent blocked depolarization-induced spermidine release, matching the pharmacological profile reported for mechanosensitive cation channels in oocyte membranes (Yang and Sachs, 1989, 1990). We attempted to modulate mechanosensitive cation channel activity by manipulating external osmolarity. Halving or doubling osmolarity caused oocytes to swell, or shrink, respectively (not

shown), but failed to stimulate spermidine efflux or activate significant currents.

DISCUSSION

Membrane Transport of Polyamines—The recent recognition of cytoplasmic polyamines as potent inducers of inward rectification of potassium channels by blocking the channel pore (Lopatin *et al.*, 1994, 1995; Ficker *et al.*, 1994; Fakler *et al.*, 1995) has prompted a resurgence of interest in the membrane interactions of polyamines. It is now apparent that cytoplasmic polyamines also induce inward rectification of glutamate receptor channels (Kamboj *et al.*, 1995; Koh *et al.*, 1995), and previous studies showed that extracellular polyamines can block, and may even permeate, *N*-methyl-D-aspartate receptors (Igarashi and Williams, 1995). Our recent studies of polyamine-induced rectification suggested that polyamines traverse essentially the full membrane field in order to reach the deepest blocking sites (Lopatin *et al.*, 1995), raising the possibility that significant efflux of polyamines might occur through Kir channels. Accordingly, we initially sought to demonstrate, using radioactive [^3H]spermidine as a tracer, that spermidine can actually efflux from *Xenopus* oocytes expressing Kir channels. However, it is clear from the results that permeation through HRK1 channels does not provide a substantial pathway for spermidine efflux (Figs. 1 and 2), relative to the efflux through endogenous pathways.

Although polyamine transport into and out of cells has been studied for many years, molecular mechanisms remain poorly understood (Khan *et al.*, 1994). Cellular uptake has received the most attention, and it is clear that there are high affinity uptake systems with saturation at low micromolar levels of polyamines in oocytes and other animal tissues (Khan *et al.*, 1990; Kano and Oka, 1976; Saunders *et al.*, 1989; Gilad and Gilad, 1991). There is some evidence for "transacceleration" of polyamine flux, whereby raising the transmembrane polyamine concentration paradoxically increases flux from the cis-side of the membrane, indicative of futile cycling of an exchange process (Byers *et al.*, 1990; Mackarel and Wallace, 1994). A literature survey suggests that little is known about the mechanistic basis of polyamine efflux. One reasonably consistent finding is that depolarization, or maneuvers likely to induce depolarization (*e.g.* increased extracellular $[\text{K}^+]$, stimulation of depolarizing ion currents, metabolic poisoning), tend to stimulate efflux, or to inhibit influx (Kashiwagi *et al.*, 1986; Khan *et al.*, 1992; Poulin *et al.*, 1995; Fage *et al.*, 1992, 1993; Nicolas *et al.*, 1994). This finding prompted us to systematically examine the hypothesis that spermidine efflux can occur by simple electrodiffusion. The use of *Xenopus* oocytes provides three essential technical advances for this purpose: 1) high level expression of potassium channels permitted voltage-clamping by manipulation of extracellular $[\text{K}^+]$; 2) the large size of oocytes permitted spermidine to be introduced by injection; 3) injected oocytes could be voltage-clamped using a two-microelectrode voltage-clamp. The results show for the first time that spermidine efflux is strictly dependent on E_m in the absence of external divalent cations (Fig. 4). The results obtained under two-microelectrode voltage-clamp demonstrate that the effect of changing external $[\text{K}^+]$ is entirely a consequence of the change in E_m , and not of changes in external $[\text{Na}^+]$, or $[\text{K}^+]$ *per se*. Thus the results provide direct evidence against a Na^+ or K^+ cotransport or countertransport mechanism being involved in spermidine efflux under the conditions of our experiments.

Although there is no *a priori* reason to suspect it, it might be argued that depolarization of the oocyte introduces some non-specific leakage through which spermidine efflux is occurring. However, the specific blockage of this effect by trans- Ba^{2+} or

Ca^{2+} ions argues strongly against this, and instead is consistent with efflux occurring through a membrane pore. In normal high $[\text{Na}^+]$ solutions, the oocyte is hyperpolarized, and electrodiffusive release is slow. Under these conditions, Ca^{2+} (1 mM) stimulates what appears to be a voltage-independent efflux. Similar mixed agonist/antagonist effects of divalent cations on polyamine uptake have been reported in human breast cancer cells (Poulin *et al.*, 1995).

It is not yet clear which channels constitute the pathway for spermidine efflux from oocytes. Mechanosensitive non-selective cation channels are present in oocyte membranes at high density (Yang and Sachs, 1990) and, like polyamine efflux, are inhibited by Ca^{2+} and Ba^{2+} , but not by TEA⁺ or verapamil (Yang and Sachs, 1989, 1990). Attempts to manipulate the open probability of these channels in intact oocytes were not successful, and indeed, we have found no reports of successful manipulation of mechanosensitive channels in whole cell currents in the literature. At present we may only speculate that the pathway for electrodiffusive release of spermidine from oocytes, and perhaps other cells, is through mechanosensitive, non-selective cation channels.

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