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Neomycin inhibits the calcium current of *Paramecium*

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The duration of high-K^+ stimulated backward swimming is a commonly used bioassay for estimating the amplitude of the inward calcium current of *Paramecium*. Electrophysiological analysis confirmed that concentrations of neomycin which decreased the duration of stimulated backward swimming also reduced the isolated inward calcium current. Other polycations were also effective in this bioassay and their effectiveness was correlated with the number of their positive charges. *Paramecium* is therefore a convenient model system for studying the effects of compounds such as neomycin on calcium currents as well as their mechanisms of action.

Introduction

Studies using the unicellular ciliate *Paramecium* have shown that there is a direct correlation between the duration of high-K^+ stimulated backward swimming and the amplitude of the voltage-dependent inward calcium current [1]. The reason for this is that ciliary reversal, and consequent backward swimming, is Ca^{2+} dependent. The Ca^{2+} enters through the ciliary voltage-dependent Ca^{2+} channel in response to the K^+-induced depolarization. Therefore, the duration of backward swimming can be used as a simple bioassay to estimate the activity of the voltage-dependent Ca^{2+} channel. Since this is an estimate, any suggested changes in Ca^{2+} channel activities are confirmed by voltage-clamp analysis. This behavioral bioassay has been used to screen for compounds, such as W-7, which can inhibit the voltage-dependent inward calcium current [2]. By using such a screening procedure, it was found that many different polycations inhibited high-K^+ stimulated backward swimming. Of these compounds, neomycin was found to be the most effective at the lowest concentration. Although neomycin has previously been shown to inhibit the high-K^+ stimulated backward swimming of *Paramecium* [3] and block the photoresponse in a related ciliate, *Paramecium bursaria* [4], no analysis of the effects on the calcium current have yet been described.

Neomycin is a highly charged compound (+6 at neutral pH) which, like many other aminoglycoside antibiotics, produce many side effects such as ototoxicity [5], nephrotoxicity [6] and inhibition of neuromuscular transmission [7] when administered to humans. Although the mechanisms of action of all of these side effects are not known, they all are inhibited by raising the external calcium concentration. We have found that a representative aminoglycoside, neomycin, reversibly inhibits the inward Ca^{2+} current of *Paramecium* when the external Ca^{2+} concentration is 1.0 mM but not when the external Ca^{2+} is raised to 5.0 mM.

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Materials and Methods

Stocks and culture procedures

Wild type *Paramecium caudatum,* stock G3, were cultured at room temperature (21° C to 28° C) in bacterized cerophyll medium supplemented with 5.0 mg/liter stigmasterol. This medium was bacterized with *Enterobacter aerogenes* 24 h before introducing the paramecia [8].

Solutions

The standard control solution for behavioral and electrophysiological analysis was called 4 : 1 : 1. This solution contained 4.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM Mops (4-morpholinepropanesulphonic acid) and was buffered to pH 7.1 with Tris-base (tris(hydroxymethyl)aminomethane). When recording ion currents in 4 : 1 : 1, the electrodes contained 2.0 M KCl. To record the isolated voltage-dependent inward calcium current (I_{Ca}), 2.0 M CsCl electrodes were used and the recording solution contained 4.0 mM CsCl, 1.0 mM $CaCl_2$, 1.0 mM Mops and 10 mM TEA-Cl (tetraethylammonium chloride), pH 7.1 with Tris-base. This Cs-TEA procedure blocked all of the K^+ currents to unveil the I_{Ca} in virtual isolation [2]. The basic high-K^+ stimulating solution was called 20K^+ Dryl's solution. Dryl's solution contains 1.0 mM NaH_2PO_4, 1.0 mM Na_2HPO_4, 2.0 mM trisodium citrate, and 1.5 mM $CaCl_2$ and has a pH of 6.8. The 20K^+ Dryl's solution has 20 mM KCl added. The other high-K^+ stimulating solution contained 1.0 mM Mops and 0.1 to 5.0 mM $CaCl_2$ with 7.0 to 30 mM KCl (pH 7.1 with Tris-base). The aminoglycosides to be tested were added to either the 20K^+ Dryl's solution or the other high-K^+ stimulating solution as the sulfate salts but all of the other additions were chloride salts.

Recording and perfusion

The techniques for voltage clamp and bath perfusion were the same as those described previously [2]. The electrodes had 10–40 megaohm resistances. The perfusion rate was about 10 ml per min and recording was begun within 1.0 min after perfusion. The recording bath volume was 1.0 ml. The membrane was held at −40 mV and the currents were recorded following 20-ms step depolarizations and hyperpolarizations from this level. Current and voltage traces were stored and analyzed by a Nicollet digital oscilloscope. All current measurements were background subtracted by determining the difference between the measured value and the leakage current, which was linearly extrapolated from the measured leakages near the holding level.

Behavioral assay

The duration of continuous ciliary reversal, CCR, was determined by measuring the length of time cells swam backwards in high-K^+ stimulating solutions, such as the 20K^+ Dryl's solution [1]. Cells were preincubated in the 4 : 1 : 1 solution for at least 15 min at room temperature before testing. The duration of CCR was expressed as the mean of eight determinations.

Results

Several different aminoglycosides were effective in inhibiting high-K^+ stimulated continuous ciliary reversals (CCR). The I_{50} value was defined as the concentration of the compound tested which pro-

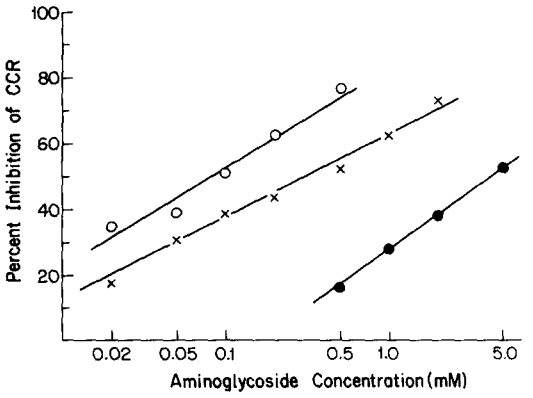

Fig. 1. Three different aminoglycosides all cause concentration-dependent inhibition of high-K^+ stimulated CCR. Neomycin (○), gentamycin (×), and spectinomycin (●) were added to the 20K^+ Dryl's solution at various concentrations and tested for their ability to inhibit high-K^+ stimulated CCR. At each concentration, the duration of CCR with the aminoglycoside was compared with the control (without aminoglycoside) so that the percent inhibition of CCR could be determined. The I_{50} values were the concentrations which were 50% effective in reducing the high-K^+ stimulated CCR. Each point is the mean of eight determinations.

duced a 50% reduction in the duration of CCR. The I_{50} values of the aminoglycosides neomycin, gentamycin, and spectinomycin were 80 μM, 300 μM and 4.2 mM, respectively (see Fig. 1). At neutral pH, neomycin has a net charge of +6, gentamycin has +4, and spectinomycin has +2. Therefore there was a correlation between the number of positive charges on the aminoglycosides tested and their I_{50} values.

This correlation was also seen with several other polycations. Spermine (+4), spermidine (+3) and Mg^{2+} had I_{50} values of 230 μM, 560 μM and 1.8 mM, respectively.

Neomycin was chosen for more detailed analysis because it had the lowest I_{50} value and was therefore the most effective compound tested. The inhibitory effects of neomycin were immediate

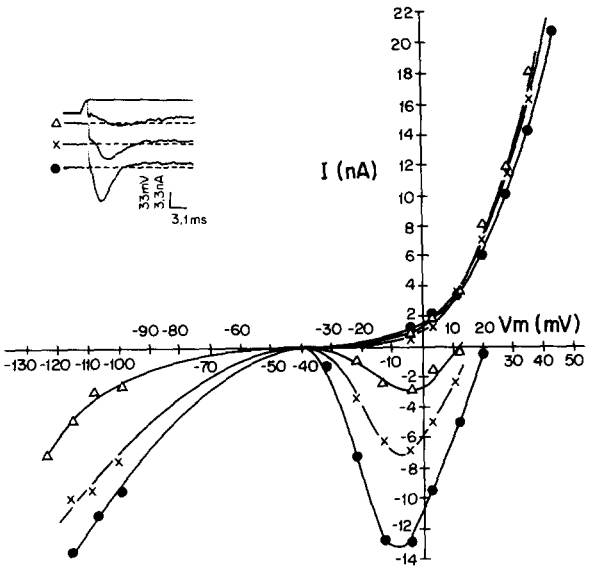

Fig. 2. Neomycin caused reductions in the measured membrane ion currents. Membrane ion currents were recorded under voltage-clamp conditions with the holding level at −40 mV. The ion currents were first measured with 20 ms voltage steps in the 4:1:1 solution (●) and then the bath was perfused with 80 μM neomycin (×) and the recordings repeated. Finally the bath was perfused with 125 μM neomycin (△) and the same measurements made. The amplitudes of the membrane ion currents (I) were plotted as a function of the voltage step applied (V). All current measurements are background subtracted. Examples of the depolarization-induced inward currents are shown in the insets. The upper trace is the voltage step used and representative current traces are shown for 4:1:1 (●), 80 neomycin (×), and 125 μM neomycin (△). This data is from one cell but it was repeated with similar results.

since there was no backward swimming in the $20K^+$ Dryl's solution when neomycin was present in concentrations of greater than 1.0 mM and this did not require any preincubation. This effect was also immediately reversible since the cells which had been tested in this 1.0 mM neomycin, $20K^+$ Dryl's solution showed normal durations of CCR when retested in $20K^+$ Dryl's alone.

Although cells were viable for hours in concentrations of neomycin as high as 2.0 mM, exposure to high concentrations of neomycin for long periods of time was toxic. After 24 h, 80% of the cells survived 0.5 mM neomycin but survival in 1.0 mM and 2.0 mM was reduced to 40% and 20%, respectively.

Several membrane ion currents, measured under voltage-clamp conditions, showed concentration-dependent changes in neomycin. When the recording solution was changed from the 4:1:1 solution to the same solution with either 80 μM or 125 μM neomycin added, decreases were reliably seen in the hyperpolarization-induced currents and the depolarization-induced transient inward current but no consistant change was seen in the voltage-dependent outward potassium current (see Fig. 2). This was not due to the sulfate since addition of equimolar Tris-sulfate did not cause any change in these currents.

The specific effects of neomycin on the hyperpolarization-induced ion currents are difficult to interpret because these ion currents have not yet been fully described in *Paramecium*. Recent work suggests that the majority of the hyperpolarization-induced ion currents seen in 4:1:1 are due to a hyperpolarization-induced calcium influx (Hennessey, in preparation) and a resulting calcium-dependent K^+ current [9]. Since all of these hyperpolarization-induced currents have not yet been isolated and characterized it is difficult to identify which may be affected by neomycin.

The depolarization-induced inward current has been well characterized and is due to a voltage-dependent calcium current, the I_{Ca} [10]. This I_{Ca} is easily isolated by blocking all of the K^+ currents with Cs-TEA (see Materials and Methods). The isolated I_{Ca} was immediately reduced to 30% of its original amplitude by bath perfusion with 125 μM neomycin and reperfusion of the bath with 4:1:1 caused the immediate return of this current

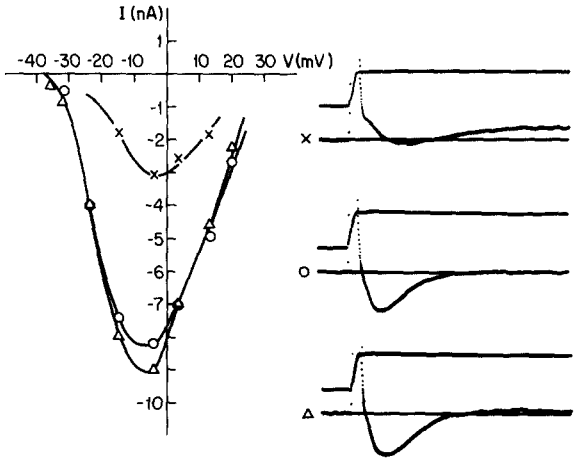

Fig. 3. The effects of 125 μM neomycin on the isolated calcium current are reversible. The I_{Ca} was isolated by blocking the K^+ currents with Cs-TEA (see Materials and Methods). The amplitudes of the I_{Ca} were plotted as a function of voltage (V) before the addition of neomycin (Δ), during exposure to 125 micromolar neomycin (×) and after removal of the neomycin (○). All current measurements were background subtracted. Representative traces of the calcium currents before (Δ), during (×) and after (○) exposure to the neomycin are shown in the inset. The top trace is the voltage step used to elicit these representative currents. Data are from one cell and are typical.

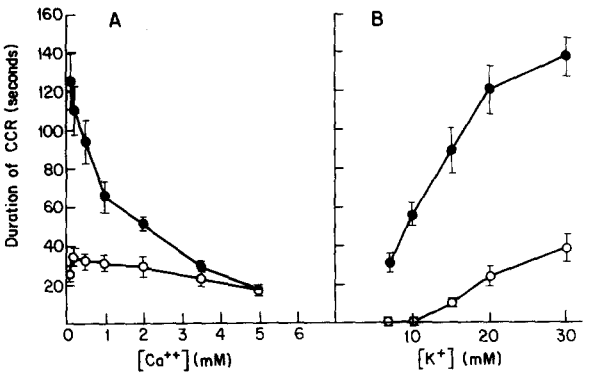

Fig. 4. (A) The duration of high K^+ stimulated CCR was reduced by 125 μM neomycin at low calcium concentrations but increasing the external calcium concentration caused a decrease in this effect. The control solutions (●) contained 30 mM K^+, 1.0 mM Mops and $CaCl_2$ at concentrations ranging from 0.1 mM to 5.0 mM (pH 7.1 with Tris-base). The neomycin solutions (○) were the same except they also contained 125 μM neomycin. Each point is the mean ± S.D. from eight measurements. (B) The duration of high-K^+ stimulated CCR was decreased by neomycin at all of the K^+ concentrations tested. The control solutions (●) were similar to those used in (A) except they contained 0.1 mM $CaCl_2$ and KCl ranging from 7.0 to 30 mM. The neomycin test solutions (○) also contained 125 μM neomycin. Each point is the mean ± S.D. from eight determinations.

to its original amplitude (see Fig. 3). Note that 125 μM neomycin caused about the same percent reduction of the duration of high-K^+ stimulated CCR (compare Figs. 1 and 3). The I_{Ca} amplitudes were also measured with cells bathed in either gentamycin, spermine, spermidine, or Mg^{2+} at the I_{50} concentrations. In all cases the I_{Ca} was rapidly and reversibly reduced by about 50% at the I_{50} concentration.

Neomycin was only effective in causing a decrease in the duration of high-K^+ stimulated CCR when the calcium concentration was low. When the external calcium concentration was increased to 5.0 mM, 125 μM neomycin was no longer effective in reducing the duration of high-K^+ stimulated CCR since there was no longer any difference between the CCR values with and without neomycin (Fig. 4A). The ability of neomycin to inhibit high-K^+ stimulated CCR was not affected by external K^+ since 125 μM neomycin was still effective in reducing the duration of CCR at external K^+ concentrations as high as 30 mM (Fig. 4B). External Ca^{2+} was apparently not limiting in these experiments because up to 130 s CCR was seen in Ca^{2+} concentrations as low as 0.1 mM (Fig. 4A).

Increasing the external calcium concentration

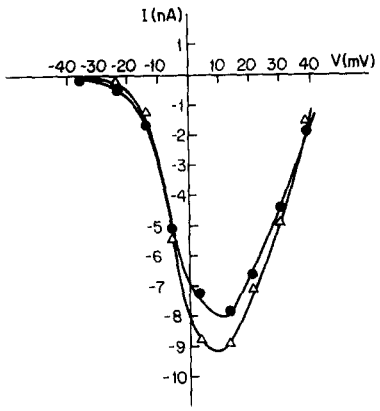

Fig. 5. Addition of 5.0 mM $CaCl_2$ caused the neomycin to be no longer effective in reducing the I_{Ca}. The I_{Ca} was isolated with Cs-TEA as in Fig. 3. The peak I_{Ca} values were first measured in 5.0 mM $CaCl_2$ (Δ) and then the bath was perfused with the same solution with 125 μM neomycin added (●) and the measurements were repeated. Data shown are from one cell and are typical.

to 5.0 mM also reduced the ability of 125 μM neomycin to inhibit the I_{Ca}. While the I_{Ca} was substantially reduced by 125 μM neomycin in 1.0 mM calcium (Fig. 3), raising the external calcium to 5.0 mM prevented this effect. The maximal I_{Ca} was the same in 5.0 mM Ca^{2+} and in 5.0 mM Ca^{2+} with 125 μM neomycin (Fig. 5). In agreement with previous observations [10], the maximum I_{Ca} values were not significantly increased in the 5.0 mM Ca^{2+} compared to 1.0 mM Ca^{2+} but the voltage at which the maximal I_{Ca} was seen was shifted towards more positive values (compared Figs. 3 and 5). In the presence of 30 mM K^+ the I_{Ca} was still reversibly reduced by 125 μM neomycin to the same extent as in Fig. 3. In summary, neomycin reduces I_{Ca} (Fig. 3) and CCR (Fig. 4A) in 1.0 mM Ca^{2+} but when the external Ca^{2+} is increased to 5.0 mM the I_{Ca} is no longer inhibited (Fig. 5). The duration of CCR is already maximally reduced in high Ca^{2+} so that an effect of neomycin cannot be measured (Fig. 4A).

Discussion

This study shows that neomycin produces a rapid, reversible inhibition of the I_{Ca} in *Paramecium*. The concentration of neomycin which is 50% effective in producing this effect is also the concentration which is the I_{50} for the behavioral effect. Similar measurements with the other polycations tested showed the same correlation. This agrees well with previous observations showing that the amplitude of the I_{Ca} is directly correlated with the duration of high-K^+ stimulated CCR [1,2]. The reliable correlation between the I_{Ca} and I_{50} values shows that the reduction of the I_{Ca} is not due to any recording artifacts. This also reconfirms the utility of behavioral bioassays for estimating Ca^{2+} channel functions in *Paramecium*.

The fact that the ability of neomycin to inhibit both the I_{Ca} and the duration of high-K^+ stimulated CCR is impaired by high external calcium suggests that the mechanism of action may have aspects in common with mechanisms involved in nephrotoxicity [11], ototoxicity [5] and blockage of neuromuscular transmission [7]. In all of these effects the potencies of the compounds tested are generally directly correlated with their number of charges at neutral pH. Much more analysis must obviously be done before a mechanism of action can be identified for neomycin's effects on the calcium current of *Paramecium*.

Although the mechanism of action and the receptor or receptors for neomycin's effects on *Paramecium* membranes have not been identified, there are three immediate possibilities: (1) A specific effect could involve the fact that neomycin binds with high affinity to phosphatidylinositol [11]. It has been suggested that phosphatidylinositol is the membrane receptor for aminoglycoside binding [6] and that such interactions are so strong that phosphatidylinositol metabolism can be inhibited [12]. This could also be involved in the cellular toxicity effects of neomycin. The high-affinity binding has been implicated in the neomycin-induced blockade of excitation-contraction coupling in muscle [13] but in this study it was not known whether inward Ca^{2+} currents were inhibited. Since neomycin is often used in such experiments to block IP_3 production and calcium-mediated events, it is important to distinguish between neomycin's effects on calcium influx and its effects on phosphatidylinositol metabolism. (2) Neomycin may be binding to Ca^{2+} channels themselves to either directly block Ca^{2+} currents or to allostericly inhibit them. (3) Neomycin may have its effects on the calcium current as a result of general interactions with negatively charged membrane species such as phospholipids, proteins, or surface sugars. In any case, the relevant neomycin binding site or sites could also be calcium binding sites as well since the neomycin effect can be negated by raising the external calcium concentration (see Figs. 4A and 5).

The role of surface charge in *Paramecium* is important because the external solution is usually very low in ionic strength, leaving the fixed negative surface charges largely unscreened. It has been proposed that screening of these negative charges by interactions with multivalent cations reduces the relatively negative surface potential without appreciably altering the measured membrane potential (V_m) [14,15]. To offset this decrease in the surface potential the 'real' or 'transmembrane' potential (E_m) may become hyperpolarized [16]. By this hypothesis, increasing external Ca^{2+} could cause a decrease in CCR

because the E_m is actually hyperpolarized as a result of surface charge screening [14–16]. Similarly, it is possible that at least part of the neomycin induced decrease in I_{Ca} and CCR is due to such surface charge screening effects. The fact that increasing external Ca^{2+} has no pronounced effect on CCR in the presence of 125 μM neomycin (closed circles, Fig. 4A) suggests that this concentration of neomycin is sufficient to largely saturate the anionic membrane sites.

The advantages of using *Paramecium* for such studies are that biochemical, behavioral, genetic, and electrophysiological analysis can all be performed in this eukaryotic unicell. This makes *Paramecium* an attractive model system for studying the mechanism of action of neomycin in respect to both it's ability to inhibit calcium currents and it's cellular toxicity. Behavioral and survival screens can be used to select for mutants with altered responses to neomycin, providing insights into possible mechanisms of action.

The fact that neomycin can inhibit calcium influx suggests that it could be used to test for the involvement of calcium influx in physiological processes. For example, neomycin inhibits the photoresponse in a related ciliate, *P. bursaria*, suggesting that calcium influx is necessary for this response [4]. Therefore the observation that compounds such as neomycin inhibit calcium currents in *Paramecium* makes such compounds interesting not only as tools for studying other physiological functions but also for gaining insights into the membrane mechanisms involved in their actions.

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