Neomycin: a specific drug to study the inositol-phospholipid signalling system?

Marc Prentki, Jude T. Deeney, Franz M. Matschinsky and Suresh K. Joseph

Department of Biochemistry and Biophysics and Diabetes Research Center, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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Neomycin, an antibiotic previously thought to interact specifically with inositol-containing phospholipids, was found to inhibit IP_3-mediated Ca^{2+} release from the intracellular stores of permeabilized insulinoma and liver cells. This inhibition could be relieved by increasing the IP_3 concentration. Radiolabelled IP_3 was found to bind tightly to columns prepared from neomycin covalently attached to glass beads. ATP was also bound by these colums. It is concluded that neomycin acts in biological systems as a weak anion exchanger and is therefore unsuitable for use as a specific tool to study the role of inositol phospholipids in intracellular signalling.

Neomycin *Aminoglycoside antibiotic* *Ca^{2+} release* *Inositol phospholipid* *Inositol phosphate*

1. INTRODUCTION

The prolonged therapeutic use of neomycin, a cationic aminoglycoside antibiotic, results in an impairment of hearing and damage to the kidneys. In studying the biochemical basis for this toxicity, Schacht and co-workers [1,2] noted that neomycin could bind tightly and selectively to polyphosphoinositides. It was also shown that neomycin interfered with the enzymatic synthesis and breakdown of these lipids [3–6].

It is now recognized that the inositol-containing phospholipids in the plasmalemma serve as precursors for at least two important intracellular messengers. These are 1,2-diacylglycerol and D-*myo*-inositol 1,4,5-trisphosphate (IP_3) whose functions respectively are to stimulate protein kinase C and to induce Ca^{2+} release from intracellular stores (review [7]). These compounds are formed as products of the phospholipase C catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). The known interaction of neomycin with PIP_2 [1,2] has provided the basis for the use of this antibiotic as a tool to study the possible involvement of PIP_2 hydrolysis in biological processes. For example, neomycin was found to suppress Ca^{2+} transients evoked by electrical excitation of skeletal muscle [8]. This observation is consistent with the proposal that IP_3 also has a messenger role in skeletal muscle and that neomycin acts to inhibit the generation of IP_3 by inhibiting phospholipase C-mediated PIP_2 breakdown [3–6,8]. The experiment, however, does not exclude the possibility that neomycin has some direct inhibitory effect on Ca^{2+} release from the sarcoplasmic reticulum [8]. Here we show that neomycin indeed exerts an inhibitory effect on IP_3-mediated Ca^{2+} release from permeabilized cells. However, this inhibition could be entirely accounted for by the ability of neomycin to bind IP_3. This aspect of the action of neomycin is discussed in relation to the utility of using this antibiotic to study signal transduction involving the inositol lipids.

2. MATERIALS AND METHODS

RINm5F insulinoma cells and rat hepatocytes were isolated as described [9,10]. The preparation of Ca^{2+} electrodes and their calibration were per-

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formed as in [11]. When IP_3-mediated $^{45}Ca^{2+}$ release was measured the hepatocytes (10 mg dry wt/ml) were permeabilized and preloaded with $^{45}Ca^{2+}$ in a medium containing 120 mM KCl, 20 mM Tris-Hepes (pH 7.2), 0.3 mM $MgCl_2$, 1.0 mM MgATP, 20 mM phosphocreatine, 10 U/ml creatine kinase, 200 μg/ml saponin, 2 μM ruthenium red, 5 μM antimycin A, 0.5 mM EGTA and 2 μCi/ml $^{45}Ca^{2+}$. Sufficient $CaCl_2$ was added to this medium to give a free $[Ca^{2+}]$ of 140 nM as determined with the Ca^{2+} electrode. After 25 min incubation at 30°C, a 0.1 ml aliquot of cells was diluted 50-fold into a medium containing 120 mM KCl and 20 mM Tris-Hepes (pH 7.2). 5 s after the dilution, the $^{45}Ca^{2+}$ remaining in the cells was measured after separation of the cells from the medium using Whatman glass-fiber filters (GF/C, 0.24 mm). The filters were washed twice with 5.0 ml of 0.25 M sucrose and counted in 15 ml of scintillation fluid (ACS II, Amersham Co.). IP_3-mediated $^{45}Ca^{2+}$ release was calculated from the difference between the counts retained on the filter in the presence and absence of IP_3 and the known specific activity of the isotope.

Neomycin B sulfate (Sigma) was reductively coupled to glass beads (CPG/CDI-Glycophase; particle size 74–125 μm; Pierce, Rockford, IL) as described by Schacht [1]. [2-^{3}H]IP_3, L-*myo*-[U-^{14}C]inositol 1-phosphate, [^{32}P]ATP and $^{45}Ca^{2+}$ were from Amersham. New England Nuclear supplied the [^{3}H]PIP_2. The IP_3 used in these studies was a generous gift from Dr Robin Irvine and was also obtained from Sigma.

3. RESULTS

To examine possible effects of neomycin on the release of Ca^{2+} induced by IP_3, the action of the antibiotic was tested in saponin permeabilized RINm5F insulinoma cells incubated in the presence of mitochondrial inhibitors. Fig.1 shows that neomycin inhibits IP_3-mediated Ca^{2+} release in a dose-dependent manner. At a low concentration of neomycin (0.25 mM; fig.1B) only the effects of submaximal concentrations of IP_3 were inhibited. When higher concentrations of neomycin were used (1.25 mM; fig.1C) the response to maximal concentrations of IP_3 was also attenuated. Fig.1 further shows that neomycin also inhibits the initial rate of net Ca^{2+} uptake. This is reflected by

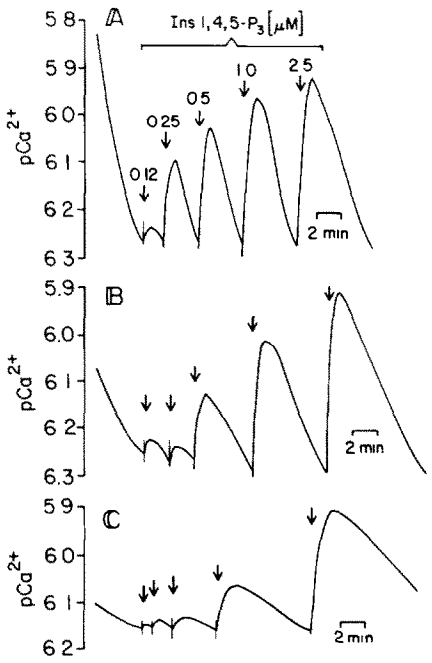

Fig.1. The effect of various concentrations of neomycin on the dose response of IP_3-mediated Ca^{2+} release. 10^6 RINm5F cells were incubated at 30°C and pH 7.0 in 0.2 ml of a buffer containing 110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 25 mM Hepes, 1 mM MgATP, 5 mM phosphocreatine, 20 units of creatine kinase/ml, 1 μg/ml oligomycin and 0.2 μM antimycin A. Saponin (50 μg/ml) was added to permeabilize the cells and the change in medium free Ca^{2+} was measured with a Ca^{2+} electrode. At the points indicated by the arrows pulse additions of IP_3 were made. From left to right these concentrations were (in μM): 0.125, 0.25, 0.5, 1.0, 2.5. (A) Control, (B) neomycin (0.25 mM), (C) neomycin (1.25 mM). Neomycin was added 6 min after cell permeabilization with saponin when the non-mitochondrial pool had lowered the medium free $[Ca^{2+}]$ to $pCa^{2+} = 5.9$.

a progressive inhibition of the amount of Ca^{2+} accumulated into the non-mitochondrial pool during the 4 min preceding the first pulse addition of IP_3 (fig.1).

Experiments of the type shown in fig.1 do not allow the effects of neomycin on Ca^{2+} uptake to be easily distinguished from its effects on IP_3-mediated Ca^{2+} release. For this reason an alternative experimental protocol was employed in which cells were pre-loaded with $^{45}Ca^{2+}$ and then diluted into medium containing IP_3 in the presence

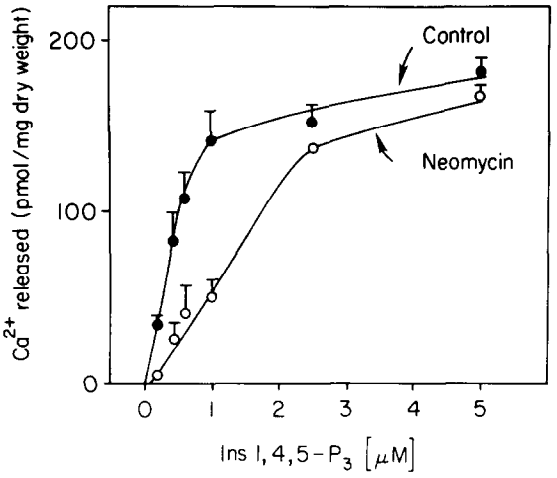

Fig.2. The influence of neomycin on IP_3-mediated $^{45}Ca^{2+}$ release from permeabilized hepatocytes. The release of $^{45}Ca^{2+}$ from pre-loaded hepatocytes was measured exactly as described in section 2. The results shown are the mean ± SE of 3 separate experiments.

or absence of neomycin. The results of these experiments, performed with rat hepatocytes, are shown in fig.2. The data obtained measuring $^{45}Ca^{2+}$ release from hepatocytes were in qualitative agreement with the Ca^{2+} electrode studies using insulinoma cells. Thus, neomycin (1.25 mM) markedly inhibited the effects of low concentrations of IP_3 but the magnitude of the inhibition diminished as the IP_3 concentration was increased.

One hypothesis that is consistent with the data in figs 1 and 2 is that neomycin (a polycationic molecule) may inhibit IP_3-mediated Ca^{2+} release simply by chelating added anionic IP_3. To test this hypothesis, columns were prepared containing neomycin covalently linked to glass beads [1]. More than 90% of the labelled IP_3 added was found to be retained by such a column (table 1). L-*myo*-Inositol 1-phosphate (IP_1) and *myo*-inositol were bound to a much lower extent whereas ATP was avidly bound. Greater than 85% of the bound IP_3 or ATP could be eluted from the column with 12 ml of 0.4 M ammonium formate/0.1 M formic acid (not shown). As shown previously [1], PIP_2 was also retained by the column (table 1). Significant non-specific binding of IP_3 can be excluded by the finding that only 11% of the IP_3 was retained by columns prepared from glass beads carried through the preparative procedure without the addition of neomycin (table 1).

4. DISCUSSION

Neomycin, a polycationic antibiotic, is generally thought to bind specifically to polyphosphoinositides and has therefore been widely used to study the involvement of this class of lipids in a variety of cellular processes. For example, it has been

Table 1

The ability of several phosphorylated compounds to bind to neomycin

	Counts loaded on column (cpm)	Counts eluting in void volume (cpm)	% bound
[^{3}H]IP_3 (1 μM)	1372	96	93
[^{14}C]IP_1 (1 μM)	10050	8760	13
myo-[^{3}H]Inositol (1 μM)	63156	51080	19
[^{3}H]PIP_2 (1 μg/ml)	994	301	70
[^{32}P]ATP (1 μM)	3672	123	97
[^{32}P]ATP (1 mM)	13359	199	98
[^{3}H]IP_3, no neomycin	1372	1219	11

Water soluble radiolabelled compounds were dissolved in 0.8 ml of 120 mM KCl and 20 mM Tris-Hepes (pH 7.2) (buffer A) containing 1 μM of the corresponding unlabelled compound. Samples were loaded onto a 0.5 ml column of neomycin-coated glass beads previously equilibrated with buffer A. The void volume was collected along with an additional 0.8 ml column wash with buffer A. [^{3}H]PIP_2 and 1 μg PIP_2 were loaded on the column dissolved in 2.0 ml $CHCl_3$:MeOH (2:1, v/v), and the void volume was collected as described above

shown to block excitation-contraction coupling in skeletal muscle [8], Ca^{2+}-dependent histamine secretion from GTP-γ-S loaded mast cells [12], thrombin-stimulated initiation of cell proliferation [13] and amylase secretion in response to carbachol from permeabilized pancreatic acini [14]. These inhibitory effects of neomycin have been used as strong evidence to link polyphosphoinositide breakdown to these different biological events. The results of this study demonstrate additional effects of neomycin that are distinguished from its action on phospholipids. Using two different cell types (insulinoma and hepatocytes), we observed that neomycin markedly inhibits IP_3-induced Ca^{2+} release from non-mitochondrial pools. In addition, MgATP-dependent uptake into these pools was also inhibited. Both these effects can be accounted for by the ability of neomycin to bind IP_3 and ATP even more avidly than phosphatidylinositol 4,5-bisphosphate. Since neomycin barely binds IP_1, it appears that the antibiotic behaves as a weak anion exchanger.

These observations raise questions regarding the specificity of this drug. It should be stressed that our data do not necessarily invalidate the conclusions drawn from previous studies where neomycin has been used as a probe to assess the involvement of inositol lipids. However, it should be recognized that neomycin has the potential to interact with polyphosphoinositides, IP_3, a variety of nucleotide triphosphates including ATP and possibly other polyanions. Thus, the usefulness of neomycin as a specific drug to study polyphosphoinositide metabolism is questionable. The above results also cast doubts that the nephro- and ototoxicity of the aminoglycosides can be simply explained by an interaction with the polyphosphoinositides.

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