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EVIDENCE THAT NEOMYCIN INHIBITS PLASMA MEMBRANE Ca^{2+} INFLOW IN ISOLATED HEPATOCYTES

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Abstract—The effects of neomycin on Ca^{2+} fluxes and inositol polyphosphates in hepatocytes were investigated since it has been proposed that this antibiotic inhibits inositol 1,4,5-triphosphate formation in fibroblasts [D. H. Carney, D. L. Scott, E. A. Gordon and E. F. LaBelle, *Cell* **42**, 479 (1985)]. In hepatocytes incubated at 1.3 mM extracellular Ca^{2+} ($Ca^{2+}{}_0$) neomycin (2 mM) inhibited $^{45}Ca^{2+}$ exchange both in the presence or absence of vasopressin. At 1.3 mM $Ca^{2+}{}_0$, but not at higher concentrations of $Ca^{2+}{}_0$, the antibiotic (2 mM) inhibited the increase in glycogen phosphorylase *a* activity observed at late but not at early times after addition of vasopressin. The antibiotic also inhibited the increase in phosphorylase activity caused by the subsequent addition of 1.3 mM $Ca^{2+}{}_0$ to cells previously incubated in the presence of vasopressin and in the absence of added $Ca^{2+}{}_0$. The concentration of the antibiotic (2 mM) which gave half-maximal inhibition of phosphorylase activation by vasopressin had no effect on the activation of phosphorylase by glucagon or the release of Ca^{2+} from intracellular stores induced by vasopressin. At a concentration of 10 mM, neomycin caused a 50% inhibition of the formation of [^{3}H]inositol polyphosphates induced by vasopressin. It is concluded that neomycin, at concentrations which inhibit phosphoinositide-specific phospholipase C in other types of cells inhibits the inflow of Ca^{2+} across the plasma membrane but does not inhibit inositol trisphosphate formation in hepatocytes.

In 1982, Quist [1] showed that neomycin, a cationic aminoglycoside antibiotic in common use, inhibits the hydrolysis of phosphoinositides in red blood cells. Subsequently, the antibiotic has been used by some investigators as a tool in studies of the role of inositol polyphosphates in red blood cells [1], fibroblasts [2], skeletal muscle [3], insulinoma cells [4], platelets [5] and hepatocytes [4, 6]. These studies have generally used neomycin at a concentration of 2 mM [1–6]. In liver cells neomycin has been shown to inhibit the increase in intracellular Ca^{2+} induced by vasopressin and epidermal growth factor [6] and, in permeabilised hepatocytes, the ability of inositol 1,4,5-trisphosphate to release Ca^{2+} from intracellular stores [4]. The mechanism by which neomycin inhibits agonist-induced increases in intracellular free Ca^{2+} and its effects on phosphoinositide hydrolysis in the liver cell have not been investigated.

The aim of the present experiments was to characterise the effects of neomycin on Ca^{2+} fluxes and inositol polyphosphate formation in isolated rat hepatocytes. The results have shown that this antibiotic inhibits Ca^{2+} inflow across the liver cell plasma membrane, but has little effect on the levels of inositol polyphosphates or the release of Ca^{2+} from internal stores.

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† Abbreviations used: InsP, *myo*-inositol monophosphate; $InsP_2$, *myo*-inositol bisphosphates; $InsP_3$, *myo*-inositol trisphosphates; and $[Ca^{2+}]_0$, concentration of extracellular Ca^{2+}.

MATERIALS AND METHODS

Materials. Neomycin sulphate (90–95% neomycin B; 5–10% neomycin C) was obtained from the Sigma Chemical Co. (St. Louis, MO), [U-^{14}C]glucose-1-phosphate, [^{3}H]*myo*-inositol and ACS II from Amersham (Australia) Pty. Ltd. (Surrey Hills, N.S.W., Australia), and AG1-X8 (100–200 mesh) resin from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of the highest grade available and were obtained from the sources described previously [7, 8].

Methods. The isolation of hepatocytes from fed male rats and incubation of the cells at 37° were performed as described previously [7, 8]. The incubation medium contained 117 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 20 mM 2-([2-hydroxy-1,1,-bis-(hydroxymethyl)ethyl]amino)ethansulphonic acid (TES)-NaOH, 1.3 mM $CaCl_2$ (except where indicated otherwise) and hepatocytes (2×10^6 cells (30 mg wet wt) per ml). Rates of plasma membrane Ca^{2+} inflow were estimated by (a) measurement of the initial rate of $^{45}Ca^{2+}$ exchange [7], (b) measurement of the increase in glycogen phosphorylase activity which persists after the release of Ca^{2+} from intracellular stores following vasopressin addition [9–13], and (c) measurement of the increase in phosphorylase activity which follows the addition of Ca^{2+} to cells previously incubated in the absence of added Ca^{2+} [13–15]. Glycogen phosphorylase *a* activity was estimated using [U-^{14}C]glucose-1-phosphate [16] as described previously [15]. Enzyme activity was expressed as μmol [^{14}C]glucose-1-phosphate incor-

porated into glycogen per min per g wet wt of cells (1 unit). The preparation of hepatocytes labelled with [^{3}H]*myo*-inositol and measurement of inositol mono-, bis- and tris-phosphates labelled with [^{3}H]inositol were performed as described previously [17].

Except where indicated otherwise, the results are the means ± SEM of the number of experiments indicated. Degrees of significance were determined using Student's *t*-test for paired samples. Values of $P > 0.05$ were considered to be not significant.

RESULTS

In cells incubated in the presence of 1.3 mM extracellular Ca^{2+} ($Ca^{2+}{}_0$) neomycin inhibited by 25% the rate of $^{45}Ca^{2+}$ exchange (Fig. 1). In the presence of vasopressin, neomycin inhibited the increase in the rate of $^{45}Ca^{2+}$ exchange caused by vasopressin [15] by 25% (Fig. 1).

Neomycin (2 mM) did not inhibit the initial increase in glycogen phosphorylase *a* activity caused by vasopressin (Fig. 2A). However, at later times a marked reduction in the response to vasopressin was observed (Fig. 2A). Similar results were obtained when the time for which neomycin was present before the addition of vasopressin was increased from 1 (Fig. 2A) to either 10 or 20 min (results not shown), and when the concentration of neomycin was increased from 2 to 10 mM (results not shown). The inhibitory effect of neomycin was abolished by increasing the $[Ca^{2+}]_0$ (Table 1). In the absence of vasopressin, neomycin decreased glycogen phosphorylase activity by 40% (Fig. 2A inset). Neomycin had no effect on (a) the increase in glycogen phosphorylase observed following the addition of glucagon (Fig. 2B), and (b) cell viability as assessed by trypan blue exclusion. For cells exposed to 2 mM neomycin for periods of up to 20 min, 89 ± 2% excluded trypan blue, compared with 87 ± 1% for control cells (data mean ± SEM of 4 experiments).

Neomycin caused an inhibition of both the initial rate and the extent of the increase in glycogen phosphorylase caused by addition of $Ca^{2+}{}_0$ to cells previously incubated in the absence of added $Ca^{2+}{}_0$ and in the presence of vasopressin (Fig. 3). The effects of different concentrations of the antibiotic on phosphorylase activity (measured 2 min after the addition of $Ca^{2+}{}_0$) are shown in Fig. 4. A concentration of about 2 mM neomycin gave half-maximal inhibition. Neomycin completely inhibited the increase in glycogen phosphorylase caused by the addition of 1.3 mM $Ca^{2+}{}_0$ to cells incubated in the absence of vasopressin and $Ca^{2+}{}_0$ (Fig. 5).

Neomycin (2 mM) had no effect on the shape of the time course for the release of $^{45}Ca^{2+}$ following addition of vasopressin to cells incubated in the

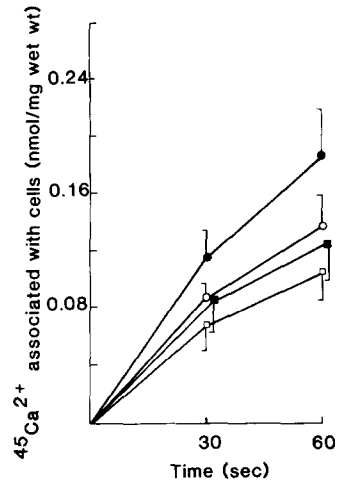

Fig. 1. Inhibition by neomycin of $^{45}Ca^{2+}$ exchange in hepatocytes. Hepatocytes were incubated in the presence of 1.3 mM $Ca^{2+}{}_0$. After incubation for 15 min either H_2O or 2 mM neomycin sulphate was added followed after 1 min by either saline or 13 nM vasopressin. After a further 10 min, tracer $^{45}Ca^{2+}$ was added to all incubations and the amount of $^{45}Ca^{2+}$ associated with the cells was determined as described in Materials and Methods. The symbols are, (○) control, (●) vasopressin, (□) neomycin and (■) neomycin plus vasopressin. The data are the means ± SEM of experiments conducted with 5 separate cell preparations. The degrees of significance are: 30 sec time point; $P < 0.01$, vasopressin compared with neomycin; 60 sec time point; $P < 0.05$, vasopressin compared with control; $P < 0.02$, neomycin compared with control; $P < 0.01$, vasopressin compared with neomycin and vasopressin compared with neomycin plus vasopressin.

Table 1. Effect of increases in the extracellular Ca^{2+} concentration on the inhibition by neomycin of vasopressin-stimulated glycogen phosphorylase *a* activity*

Extracellular Ca^{2+} concentration (mM)	Activity of glycogen phosphorylase *a* (units)	
	Saline	Neomycin
1.3	26.3 ± 1.4	18.7 ± 0.8†
2.6	24.4 ± 1.4	22.7 ± 1.0
5.3	25.2 ± 1.2	24.3 ± 1.1
10.6	24.2 ± 0.7	22.4 ± 0.7

* Hepatocytes were incubated in the presence of the indicated $[Ca^{2+}]_0$ as described in the legend of Fig. 2. Glycogen phosphorylase *a* activity was measured at 10 min after the addition of vasopressin. The data are the means ± SEM of experiments conducted with 7 (1.3 mM Ca^{2+}) or 3 (other Ca^{2+} concentrations) cell preparations. The degree of significance for comparisons between values obtained in the presence of saline and neomycin is † $P < 0.001$.

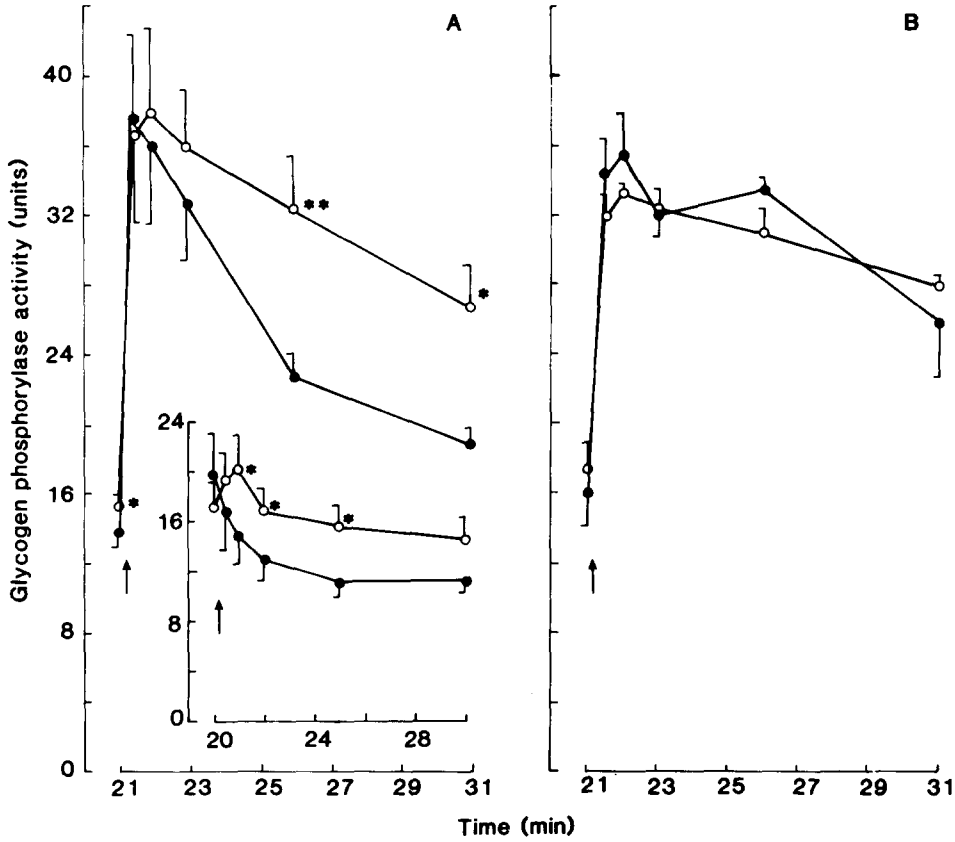

Fig. 2. Effect of neomycin on the stimulation by vasopressin or glucagon of glycogen phosphorylase *a* activity. Hepatocytes were incubated in the presence of 1.3 mM $Ca^{2+}{}_0$. At 20 min either saline (○) or 2 mM neomycin sulphate (●) was added, followed by 13 mM vasopressin (A) or 170 nM glucagon (B) at 21 min (indicated by the arrow). The inset in (A) shows the effect of neomycin alone. At the arrow either saline (○) or 2 mM neomycin sulphate (●) was added. The data are the means ± SEM of experiments conducted with 4(A) or 3(A inset or B) separate cell preparations. The degrees of significance for comparisons between values obtained in the presence of saline and neomycin are * $P < 0.05$ and ** $P < 0.02$.

presence of 0.1 mM $^{45}Ca^{2+}{}_0$ and did not alter the maximum amount of $^{45}Ca^{2+}$ released (Fig. 6). At 10 mM, neomycin inhibited by 50% the formation of inositol polyphosphates induced by 10 min incubation with vasopressin (Table 2). In the absence of the hormone, a small increase in [^{3}H]$InsP_3$ was observed in cells treated with neomycin alone (Table 2). The antibiotic had no effect on the increase in [^{3}H]$InsP_3$ induced by 1 min exposure to vasopressin. For cells incubated with vasopressin for 1 min in the presence of neomycin (added 20 min before vasopressin) or with the hormone for 1 min in the absence of the antibiotic, the amounts of [^{3}H]$InsP_3$ were 510 and 500% of the control, respectively.

DISCUSSION

The observations that neomycin inhibits (a) $^{45}Ca^{2+}$ exchange measured at 1.3 mM $Ca^{2+}{}_0$ in the presence of vasopressin and (b) the increase in phosphorylase caused by the subsequent addition of $Ca^{2+}{}_0$ to cells incubated in the presence of vasopressin and in the absence of added $Ca^{2+}{}_0$ (cf. [13]) indicate that the antibiotic inhibits plasma membrane Ca^{2+} inflow catalysed by vasopressin-activated Ca^{2+} transporters (cf. [7, 13]). Consistent with this conclusion are the observations that (a) in cells incubated at 1.3 mM $Ca^{2+}{}_0$ neomycin inhibits the ability of vasopressin to increase glycogen phosphorylase activity at longer, but not at shorter, times after addition of the hormone (i.e. after Ca^{2+} has been released from intracellular stores [18]) (cf. the actions of vasopressin at low [$Ca^{2+}{}_0$] [9–12, 15] or in the presence of diltiazem or verapamil [12, 13]) and (b) this inhibitory effect of neomycin is not observed at higher values of $[Ca^{2+}]_0$.

It is considered unlikely that the effects of neomycin on vasopressin action are due to displacement by the antibiotic of the hormone from its receptor since the concentration of neomycin (2 mM) which inhibited vasopressin-stimulated phosphorylase had no effect on vasopressin-stimulated $^{45}Ca^{2+}$ release. Moreover, the absence of an effect of neomycin on glucagon-stimulated phosphorylase activity indicates that the effects of the antibiotic on vasopressin-stimulated phosphorylase activity are unlikely to be due to alterations in the activities of other enzymes, including phosphatases and that the cells are not generally affected by neomycin.

In addition to its effects in the presence of vaso-

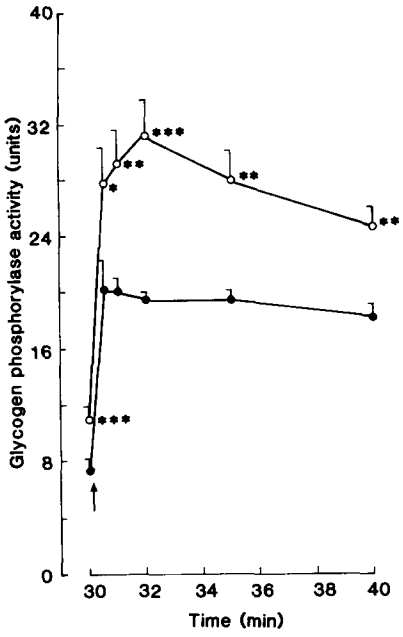

Fig. 3. Effect of neomycin on the increase in glycogen phosphorylase caused by the addition of $Ca^{2+}{}_0$ to cells previously incubated in the absence of added $Ca^{2+}{}_0$ and in the presence of vasopressin. Hepatocytes were incubated in the absence of added $Ca^{2+}{}_0$. At 20 min either saline (○) or 2 mM neomycin sulphate (●) was added, followed by vasopressin at 21 min. At 30 min, 1.3 mM $CaCl_2$ was added, (indicated by the arrow). The data are the means ± SEM of experiments conducted with 5 cell preparations. The degrees of significance for comparisons between values obtained in the presence of saline and neomycin are * $P < 0.05$; ** $P < 0.02$ and *** $P < 0.01$.

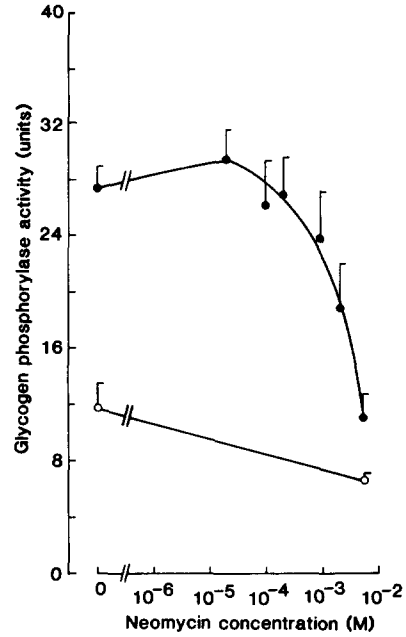

Fig. 4. Concentration dependence of the effect of neomycin on the increase in glycogen phosphorylase caused by the addition of $Ca^{2+}{}_0$ to cells previously incubated in the absence of added $Ca^{2+}{}_0$ and in the presence of vasopressin. The experiments were performed as described in Fig. 3 except that the concentration of neomycin was varied as indicated. At 21 min either saline (○) or 13 nM vasopressin (●) was added, followed by 1.3 mM $CaCl_2$ at 30 min. Samples were removed for the measurement of glycogen phosphorylase 2 min after $CaCl_2$ addition (32 min in Fig. 3). The data are the means ± SEM of 2 determinations made on each of 2 separate cell preparations.

Table 2. Effect of neomycin on vasopressin-induced increases in the amounts of [^{3}H]inositol polyphosphates in hepatocytes pre-labelled with [^{3}H]inositol*

Additions	Inositol polyphosphate measured	Radioactivity in inositol polyphosphate
Neomycin (10 mM)	InsP	98 ± 4
	$InsP_2$	99 ± 3
	$InsP_3$	178 ± 23†
Vasopressin (10 nM)	InsP	149 ± 8‡
	$InsP_2$	336 ± 40‡
	$InsP_3$	1316 ± 317†
Vasopressin (10 nM) + neomycin (10 mM)	InsP	118 ± 6†
	$InsP_2$	226 ± 25‡
	$InsP_3$	669 ± 116‡

* Hepatocytes pre-labeled with [^{3}H]inositol were incubated for 20 min in the absence or presence of 10 mM neomycin sulphate before addition of vasopressin. After a further period of 10 min, trichloracetic acid extracts were prepared and [^{3}H]-labelled inositol polyphosphates separated as described in Materials and Methods. For each inositol polyphosphate, the amount of radioactivity present in treated cells is expressed as a percentage of that present in the corresponding control incubation. In control cells, the amounts of radioactivity present in InsP, $InsP_2$, $InsP_3$ were 81,400 ± 10,600 (3), 32,300 ± 4,700 (3) and 2,100 ± 800 (3), dpm/g wet wt of cells respectively. The results are the means of SEM of 3 experiments. The degrees of significance for comparisons of treated cells with the corresponding control cells are † $P < 0.05$ and ‡ $P < 0.025$. The degree of significance for a comparison of the amount of radioactivity present in $InsP_3$ for cells treated with vasopressin with that in cells treated with vasopressin plus neomycin is $P < 0.05$.

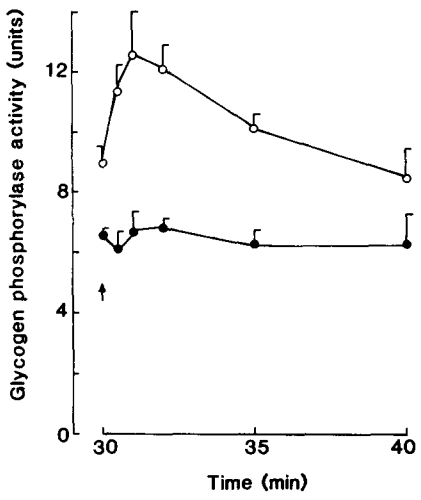

Fig. 5. Effect of neomycin on the increase in glycogen phosphorylase caused by the addition of $Ca^{2+}{}_0$, to hepatocytes incubated in the absence of vasopressin. Hepatocytes were incubated in the absence of added $Ca^{2+}{}_0$ before the addition of either saline (○) or 5 mM neomycin sulphate (●) at 20 min. At 30 min, 1.3 mM $CaCl_2$ was added (indicated by the arrow). The data are the means ± SEM of experiments conducted with 3 cell preparations.

pressin, in the absence of the hormone neomycin inhibited $^{45}Ca^{2+}$ exchange and the increase in glycogen phosphorylase activity which follows the addition of $Ca^{2+}{}_0$ to cells previously incubated in the absence of added $Ca^{2+}{}_0$. These results indicate that neomycin inhibits basal plasma membrane Ca^{2+} inflow transporters as well as those activated by vasopressin. The observation that the antibiotic had no effect on vasopressin-induced $^{45}Ca^{2+}$ release indicates that neomycin does not inhibit Ca^{2+} outflow across the plasma membrane.

It has previously been shown that hormone-stimulated Ca^{2+} release from hepatocytes requires the formation of inositol 1,4,5-trisphosphate and its subsequent action on the endoplasmic reticulum

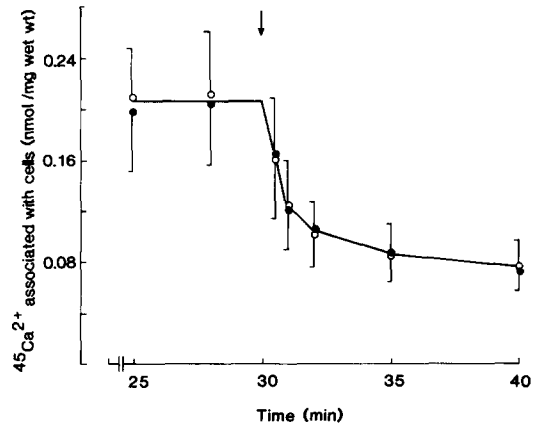

Fig. 6. Effect of neomycin on the release of $^{45}Ca^{2+}$ caused by vasopressin. Hepatocytes were incubated in the presence of 0.1 mM $^{45}Ca^{2+}$. At 29 min either saline (○) or 2 mM neomycin sulphate (●) was added, followed by vasopressin (13 nM) at 30 min (indicated by the arrow). The data are the means ± SEM of experiments conducted with 3 cell preparations.

[19, 20]. The observation that 2 mM neomycin had no effect on vasopressin-stimulated $^{45}Ca^{2+}$ release indicates that, at this concentration, the antibiotic does not inhibit the formation and action of inositol 1,4,5-trisphosphate in hepatocytes. Even 10 mM neomycin inhibited vasopressin-stimulated increases in inositol polyphosphates by only 50%. It was recently reported that neomycin, at a concentration of 2 mM, inhibited the increase in intracellular free Ca^{2+} (measured using quin2) caused by the action of vasopressin on hepatocytes, but had no detectable effects on vasopressin-induced increases in inositol polyphosphates. The present results suggest that the effects of neomycin on intracellular free Ca^{2+} [6] may be due primarily to inhibition of Ca^{2+} inflow. The difference between the effects of neomycin on hepatocytes and on other cell types [1–5] may be due to differences in the environment of the phosphoinositide-specific phospholipase C in the plasma membrane, and in the uptake and metabolism of neomycin.

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