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NEOMYCIN AND SPERMINE HAVE SIMILAR EFFECTS ON SECRETION AND PHOSPHOINOSITIDE METABOLISM IN ATP-DEPLETED PERMEABILIZED ADRENAL CHROMAFFIN CELLS

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To evaluate the requirement of bovine adrenal chromaffin cells for inositol phospholipids in secretion, the effects of neomycin and spermine on secretion and phosphoinositide metabolism were compared. Spermine and neomycin had virtually identical effects on secretion and phosphoinositide levels. Both polyamines 1) partially maintained secretion when added to permeabilized cells in the absence of ATP, 2) had no effect when added to cells in the presence of ATP and 3) inhibited secretion when present with the Ca^{2+} stimulus. In the absence of ATP the enhancements to secretion due to incubation with either polyamine were associated with sustained levels of the polyphosphoinositides. The ability of spermine and neomycin to maintain secretion and the polyphosphoinositides in the absence of ATP supports the hypothesis that the maintenance of the polyphosphoinositides is necessary for secretion. Neomycin was found to inhibit Ca^{2+} stimulated production of both inositol *bis*- and *tris*-phosphates while spermine was found to selectively inhibit inositol *bis*-phosphate production and had no effect on Ca^{2+}-stimulated inositol *tris*-phosphate production in permeabilized cells.

Calcium dependent secretion in digitonin permeabilized chromaffin cells in monolayer culture is sustained by ATP (1,2,3). The ATP dependence was proposed to be due, in part, to maintenance of the polyphosphoinositide phospholipids (4). Eberhard et al. (4) suggested that the polyphosphoinositides are needed for exocytosis in a capacity other than as substrates for a Ca^{2+} or guanine nucleotide stimulated polyphosphoinositide phospholipase C activity. In that study, the secretory response was found to be correlated with endogenous polyphosphoinositide levels. Eberhard and Holz (5) recently described a Ca^{2+}-stimulated increase in inositol lipid phosphorylation in both digitonin permeabilized chromaffin cells and intact cells stimulated by nicotinic agonist or K^+-induced depolarization. The present study provides further evidence that sustaining the polyphosphoinositides is a function of ATP in catecholamine release from this tissue.

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Abbreviations: EMEM, Eagle's Minimal Essential Medium; KGEP, Potassium Glutamate-EGTA-PIPES solution; Ins*P*, L-*myo*-inositol phosphate; InsP_2, L-*myo*-inositol bis(phosphate); InsP_3, L-*myo*-inositol tris(phosphate); PtdIns, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol; PtdIns*P*, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol phosphate; PtdInsP_2, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol bis(phosphate); PIPES, piperazine-N,N'-*bis*(2-ethanesulfonic acid); PSS, Physiological Salt Solution.

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The aminoglycoside antibiotic, neomycin, inhibits polyphosphoinositide phosphatases and inositol lipid specific phospholipase C activities (4,5,6). Although neomycin has been observed to inhibit Ca^{2+}-dependent secretion from bovine chromaffin cells (7), Eberhard et al. (4) found that neomycin could be used to increase Ca^{2+}-dependent, ATP-independent secretion. In that study, cells were exposed to neomycin only during the permeabilization step and neomycin was rinsed out before the subsequent Ca^{2+} incubation. In the absence of exogenous ATP, neomycin pretreatment was found to augment subsequent Ca^{2+}-dependent secretion. Thus, neomycin was found to have at least two distinct effects on the secretory response in chromaffin cells: 1) neomycin partially maintained Ca^{2+}-dependent secretion in the absence of ATP and 2) neomycin directly inhibited secretion when present with Ca^{2+}.

Recent reports of the effects of polyamines on inositol phospholipid metabolism (8,9,10,11,12) prompted this study on the effects of spermine on Ca^{2+}-dependent secretion from bovine adrenal chromaffin cells. In the present study, we compared the effects of spermine to those of neomycin on secretion, polyphosphoinositides, and production of inositol phosphates. We propose that the effects of these two polycationic amines on Ca^{2+}-dependent secretion is directly related to their interaction with the polyphosphoinositides. Because spermine is a naturally occurring polyamine it may influence the secretory pathway *in vivo*.

Materials and Methods

Chromaffin Cell Culture and [^{3}H]Inositol Labeling - Chromaffin cells were isolated by dissociation of bovine adrenal medullae, purified by differential plating (13), and cultured as previously described (14) in collagen coated 96-well dishes. Experiments were performed 4-8 days after culture preparation. Cellular inositol-containing lipids were labelled by incubating cells with 20 μCi/ml *myo*-[2-^{3}H]inositol in EMEM to isotopic equilibrium as described (4). Experiments were initiated after washing each well three times with PSS and permeabilizing the cells with 20 μM-digitonin in KGEP containing 139 mM potassium glutamate, 5 mg/ml bovine albumin, 20mM-PIPES (pH 6.6), 5 mM EGTA and various amounts of Ca^{2+} to give free Ca^{2+} concentrations in the micromolar range. Ca^{2+} concentrations were calculated with the computer program of Chang et al. (15), by using the constants of Portzehl et al. (16). Other components of the KGEP solutions are detailed in the figure legends. The lipids were extracted as described (4) and the deacylation products were prepared and separated as described by Downes & Michell (17). [^{3}H]Inositol phosphates were extracted as described (4) and separated by anion-exchange chromatography as described by Berridge et al. (18).

Secretion Assay and Cell Permeabilization - Catecholamine secretion was measured by prelabelling catecholamine stores in chromaffin cells with [^{3}H]noradrenaline as described (19). Cells were washed for 5 min three times with PSS before experimental treatments. Cells were permeabilized in KGEP containing 20 μM-digitonin and 5 mg/ml bovine albumin (fraction V, heat inactivated one hr at 75°C). Ca^{2+} was buffered at 10 μM with EGTA and Mg^{2+} and MgATP were present as indicated in the figure legends. Ca^{2+}-stimulated secretion was stopped by removing the test solution and the unreleased ^{3}H-norepinephrine was suspended in 1% Triton X-100 and counted separately.

Data Analysis - Data are expressed as means +/- S.E.M. with 3 replicates per group. Ca^{2+}-dependent secretion was calculated as the difference between the percentages of loaded ^{3}H released in the presence and in the absence of 10 μM-Ca^{2+}. The standard error of the mean (S.E.M.) of the difference between equal-sized groups 1 and 2 was $(S.E.M._1^2 + S.E.M._2^2)^{1/2}$. Error bars smaller than symbols were omitted from the figures.

Materials - Cell culture medium was purchased from Whittaker Bioproducts (Walkersville, MD) and fetal-bovine serum was purchased from HyClone Laboratories

(Logan, UT). Neomycin-SO_4 and spermine-Cl were obtained from Sigma Chemical Company (St. Louis, MO). *myo*-[2-^{3}H]Inositol (1 mCi/ml, 15 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO); polar contaminants were removed before use by adding 10% volume (100μl) packed AG 1-X8 anion exchange resin (BioRad Laboratories, Richmond, CA) to the aqueous [^{3}H]inositol. [^{3}H]Noradrenaline was from Amersham Corporation (Arlington Heights, IL). All other reagents were obtained from standard commercial sources in the U.S.A.

RESULTS

In the absence of ATP and neomycin, the levels of both [^{3}H]PtdIns*P* and [^{3}H]PtdInsP_2 decreased by 50% or more in digitonin permeabilized chromaffin cells (Figure 1). In the presence of ATP, the lipid levels did not change in the absence of the polyamines. When neomycin was present at or above 30 μM in the presence of ATP, both inositol lipids accumulated to levels above that present in the intact cell (time zero).

In the absence of ATP, neomycin treatment significantly increased PtdInsP_2 levels and prevented the decrease in PtdIns*P* that occurred in the absence of ATP. Spermine affected chromaffin cell polyphosphoinositides in a similar manner to neomycin (Figure 2). As observed for neomycin, the spermine effect was more pronounced on PtdInsP_2 than PtdIns*P*. Neither neomycin nor spermine promoted

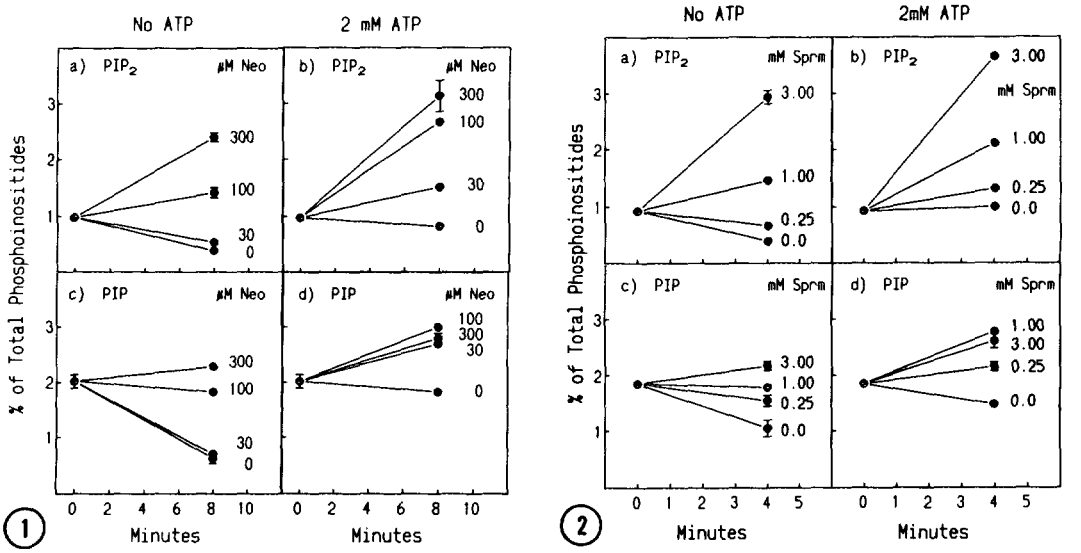


Figure 1. Neomycin increased inositol phospholipids in permeabilized cells. Chromaffin cells were labeled in EMEM containing 20 μCi [^{3}H]inositol per ml for 54 hours. The cells were permeabilized with 20 μM digitonin in KGEP containing the indicated amount of neomycin-SO_4 and with or without 2 mM MgATP. After 8 minutes the test solution was removed and the phospholipids were extracted with 100 μl ice cold methanol as described in the methods section.

Figure 2. Spermine increased inositol phospholipids in permeabilized cells. Chromaffin cells were labeled in EMEM containing 20 μCi [^{3}H]inositol per ml for 42 hours. The cells were permeabilized with 20 μM digitonin in KGEP containing the indicated amount of spermine-Cl and with or without 2 mM MgATP. After 4 minutes the test solution was removed and the cellular phospholipids were extracted with 100μl ice cold methanol as described in the methods section. The chloride content during permeabilization was kept constant at 12 mM with KCl (assuming 4 mole chloride per mole spermine).

significant changes in the size of the PtdIns pool which normally comprised greater than 90% of the inositol lipid pool (Table 1). The amount of label in the total pool of measured inositol lipids did not change significantly over the time courses of the experiments in response to polyamine treatment. Treatment with 10 μM Ca^{2+} caused the ^{3}H-cpm in the combined inositol lipid fractions (PtdIns + PtdIns*P* + PtdInsP_2) to decrease by 10% in both the control and the polyamine treated groups. This decrease along with changes in the polyphosphoinositides has been shown in other experiments to account quantitatively for the production of inositol phosphates (ref 20).

Neomycin present with Ca^{2+} inhibited the Ca^{2+}-dependent production of both InsP_2 and InsP_3 (Table 1). In contrast, spermine selectively inhibited Ca^{2+}-dependent InsP_2 production and had no effect on InsP_3 production. Neither polyamine significantly altered the level of Ca^{2+} stimulated Ins*P* production suggesting that the polyamines did not interact with PtdIns. If neomycin was rinsed from the cells, the pretreatment caused a 10-fold increase in the subsequent Ca^{2+}-stimulated production of inositol phosphates in the absence of ATP, and a 1.3-fold increase in the presence of ATP (Table 2). Thus, the inhibitory effect of neomycin on inositol lipid hydrolysis was readily reversed.

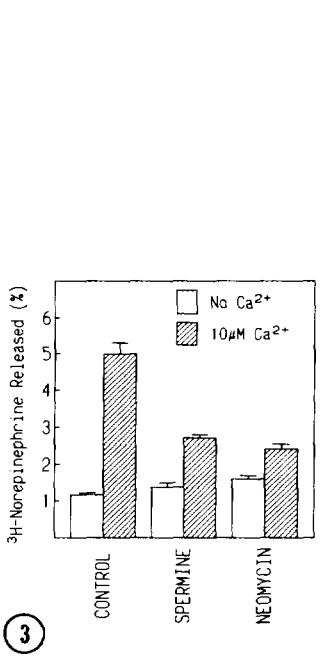
Both neomycin and spermine inhibited Ca^{2+}-dependent secretion in permeabilized chromaffin cells when present with Ca^{2+} (Figure 3). In the absence of ATP, both polyamines partially reversed the inhibition of the secretory response if they were rinsed from the cells before Ca^{2+} was added (Figure 4). In the presence of ATP neither polyamine was stimulatory or inhibitory when rinsed out before Ca^{2+}.

Table 1. Effects of Neomycin and Spermine on phosphoinositides and inositol phosphates. [^{3}H]Inositol labelled cells were permeabilized in KGEP with 2 mM MgATP for 4 min. A second incubation for 8 min contained MgATP in KGEP with or without calcium. Where indicated, polyamine was present in both incubations.

Treatment Group	Free Ca^{2+} (μM)	CPM (s.e.m.)			CPM (s.e.m.)		
		PtdIns	PtdIns*P*	PtdInsP_2	Ins*P*	InsP_2	InsP_3
Control	0	38118	892	393	170	34	40
		(1320)	(19)	(5)	(17)	(3)	(6)
	10	33401	1033	359	526	320	271
		(3546)	(93)	(51)	(13)	(58)	(13)
Spermine	0	36974	1391	1631	153	22	47
2 mM		(384)	(75)	(85)	(12)	(3)	(9)
	10	33312	1409	1329	475	173	333
		(672)	(69)	(99)	(20)	(15)	(30)
Neomycin	0	36430	1549	1235	235	35	45
200 μM		(721)	(97)	(49)	(21)	(2)	(3)
	10	30176	1740	1119	521	155	127
		(1228)	(6)	(74)	(29)	(7)	(3)

Table 2. Production of Inositol Phosphates after pretreatment with neomycin before calcium stimulation. [^{3}H]Inositol labelled cells were permeabilized in KGEP for 4 min with neomycin or ATP or both. Cells were then rinsed for 4 min without neomycin in the continuing presence or absence of ATP. A third incubation was the same as the second except for the presence or absence of calcium. The CPM are the sum of InsP + InsP_2 + InsP_3.

Treatment	CPM (s.e.m.) 0 μM Ca^{2+}	10 μM Ca^{2+}
Control	147	212
	(8)	(6)
Neomycin	366	1038
200 μM	(4)	(33)
MgATP	268	1722
2 mM	(4)	(194)
Both	406	2356
	(8)	(68)



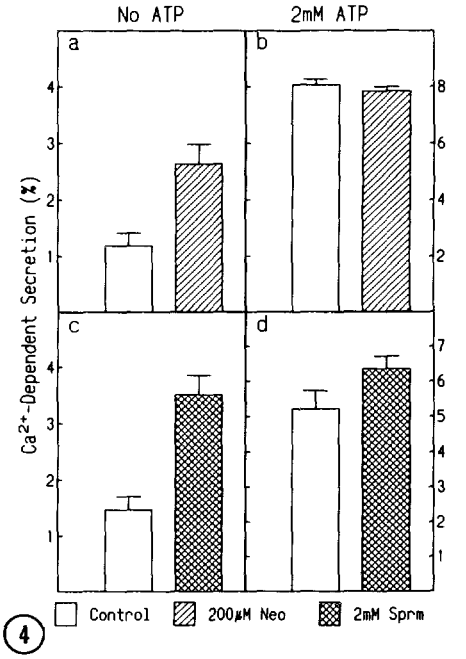


Figure 3. Spermine and neomycin inhibited Ca^{2+}-dependent secretion. Cells were loaded with [^{3}H]noradrenaline for 6 hours. Cells were permeabilized in KGEP with 20 μM digitonin, 2 mM MgATP, 0.5 mM MgCl2, and either 2 mM spermine or 200 μM neomycin. After 8 min the solution was changed to KGEP with or without Ca^{2+} and the same amount of spermine or neomycin. Secretion was stopped at 4 min as described in the methods section.

Figure 4. Polyamines partially prevented the time dependent loss of secretory response in the absence of ATP. Cells were loaded with [^{3}H]noradrenaline and permeabilized for 4 min in the presence of either 0.5 mM $MgCl_2$ (panels a and c) or 2 mM MgATP (panelsb and d) and either 2 mM spermine (panelsc and d) or 200 μM neomycin (panelsa and b). All samples were rinsed for 4 minutes with KGEP and constant MgATP or $MgCl_2$ and without spermine or neomycin. The rinse solution was replaced with KGEP without spermine or neomycin and with or without 10 μM-Ca^{2+} for 4 min.

The effect of other amines of the ornithine (putrescine and spermidine) and lysine (cadaverine) pathways were examined at a concentration of 2 mM in permeabilized cells. Only spermine had a stimulatory effect on Ca^{2+}-dependent secretion in the absence of exogenous ATP (data not shown).

DISCUSSION

MgATP is required for a secretory response sustained over time (*in vitro*) in permeabilized chromaffin cells (19,23,24). Intact cells are probably primed by intracellular ATP so that immediately upon permeabilization, the initial secretion response is independent of added MgATP (1,2,3). Holz et al. (1) showed that MgATP partially maintained the primed state after permeabilization by acting before Ca^{2+} in the secretory pathway. A separate study suggested that ATP maintained the secretory response, in part, through the maintenance of the polyphosphoinositides through PtdIns- and PtdIns*P*-kinase activities (4). The study showed that PtdIns*P* and PtdInsP_2 probably functioned in a capacity independent of inositol phosphate derivative(s) or diacylglycerol production. Neomycin was used in (4) to manipulate phosphoinositide levels to support the conclusion that the inositol phospholipids were necessary for the secretion response.

The purpose of the present study was to characterize the effects of neomycin on secretion and polyphosphoinositide levels and compare the neomycin effects to those of the naturally occurring polyamine, spermine. The effects of both neomycin and spermine support the conclusions of Eberhard et al. (4). In the absence of exogenous ATP, neomycin and spermine partially protected the loss of the initial rapid component of secretion when incubated with cells before stimulation with Ca^{2+} (Figure 4). The partial protection of the secretory response was associated with the maintenance of the polyphosphoinositides (Figures 1 and 2). In the presence of ATP, when polyphosphoinositide levels did not fall (Figures 1 and 2), preincubation with the polyamines did not alter secretion (Figure 4).

The polyamines were able to maintain a limited portion of the secretory response probably because 1) the loss of cytosolic factors during the eight minutes of treatment and rinsing and 2) exogenous polyamine that was not completely rinse out may have inhibited a portion of the secretory response. The inhibitory effects of polyamines on secretion in the presence of Ca^{2+} was possibly due to the polycations binding to PtdIns*P* and/or PtdInsP_2 and preventing their utilization by the secretory apparatus. The data in this work support this hypothesis in that 1) PtdIns*P* and PtdInsP_2 did not decrease in the absence of ATP when the polyamine was present and 2) removal of the polyamines restored a portion of the secretory response. Furthermore, removal of neomycin from cells before Ca^{2+} treatment resulted in a 10-fold increase in Ca^{2+}-dependent inositol phosphates produced in the absence of ATP and a 1.3-fold increase in the presence of ATP. Thus, the inhibitory effect on the production of inositol phosphates was reversible. The polyamines maintained or increased the polyphosphoinositides in the absence or presence of ATP, thereby providing more substrate for subsequent Ca^{2+}-dependent hydrolysis. Additional studies on the binding sites for spermine and neomycin in chromaffin cells are warranted to evaluate this model. Alternatively, the polyamines may have directly inhibited a different structural or enzymatic component of the secretory apparatus that utilizes Ca^{2+}.

Neomycin and spermine probably maintained the polyphosphoinositides, at least in part, by inhibiting inositol lipid phosphatases due to polyamines binding to the negatively charged lipids. A puzzling effect of both neomycin and spermine was the increase in the levels of PtdInsP_2 in the absence of exogenous ATP. The accumulation of PtdInsP_2 in the absence of exogenous ATP may have resulted from residual cytosolic ATP, an unaccounted for store of endogenous ATP or some other phosphate donor. PtdInsP_2 production may have been enhanced by direct stimulation of PtdIns$P$ kinase by polyamines which has been reported (9,21,22). In the presence of ATP, neomycin and spermine increased PtdIns$P$ and PtdInsP_2 above the levels found in intact cells but did not enhance secretion. This result suggested that super-normal levels of the phosphoinositides did not affect the secretory response and the secretion apparatus was saturated at normal cellular levels of the lipids.

An important question is whether endogenous spermine plays a modulatory role on the levels of the polyphosphoinositides or Ca^{2+}-dependent secretion. The lowest concentration of spermine which had effects, 0.25 mM, is in the concentration range for endogenous spermine, although this has not been measured in chromaffin cells. While fibroblasts may have as much as 0.64 mM spermine, erythrocytes may have only 15 μM spermine (calculated by Morgan (25)). The high concentrations of spermine required to increase the polyphosphoinositides may have been necessitated by the relatively short incubation times (4 min in some experiments). Chronic exposure to endogenous concentrations of spermine, *in vivo*, may maintain the polyphosphoinositides while having insignificant direct inhibitory effects on Ca^{2+}-dependent secretion. Spermine may also buffer the polyphosphoinositides from changes when cells suffer loss of ATP due to metabolic alterations. Modulation of the levels of the polyphosphoinositides by spermine in intact cells could also affect agonist stimulated InsP_3 and DAG production as well as directly modulate secretion.

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