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Neomycin inhibits K^+- and veratridine-stimulated noradrenaline release in rat brain slices and rat brain synaptosomes

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The possible involvement of phosphoinositides' turnover in the process of neurotransmitter release in the central nervous system (CNS) was studied using rat brain slices and synaptosomes. A depolarizing concentration of potassium chloride (25 mM) induces an 8.6 ± 0.4% increase of [^{3}H]noradrenaline ([^{3}H]NA) fractional release in cerebral cortical slices above spontaneous release, and 15 mM KCl induces a 3-fold increase of [^{3}H]NA release in rat brain synaptosomes. Neomycin, an aminoglycoside which binds phosphoinositides, inhibits the potassium-induced release in cortical slices with an $IC_{50} = 0.5 \pm 0.07$ mM and with $IC_{50} = 0.2 \pm 0.03$ mM in synaptosomes. Veratridine, a veratrum alkaloid which increases membrane permeability to sodium ions and causes depolarization of neuronal cells, induces a net 13.4 ± 0.3% increase of [^{3}H]NA fractional release above spontaneous release in cortical slices. In analogy to K^+ stimulaton, neomycin inhibits the veratridine-stimulated release in cortical slices with an $IC_{50} = 0.65 \pm 0.1$ mM. It appears that the recycling of phospoinositides, which is necessary for Ca^{2+} mobilization, participates in the Ca^{2+}-dependent induced neurotransmitter release in the central nervous systen.

Release; Inositol triphosphate; Noradrenaline; Neomycin; (Rat brain slice)

1. INTRODUCTION

Neomycin, a polycationic antibiotic complex with nephrotoxic and ototoxic properties, interacts selectively with anionic phospholipids [1-4].

This aminoglycoside interacts specifically with phosphatidylinositol monophosphate (PIP) and PIP_2, and inhibits their metabolism [5-8]. Neomycin was shown to decrease the amount and the ^{32}P-labeling of renal phosphatidylinositol bisphosphate [7] and to inhibit the carbamylcholine breakdown of phosphoinositides in membranes prepared from neonatal rat pancreatic islet cells [8]. Recently, neomycin was shown to inhibit thrombin-stimulated inositol phosphate formation in human platelets [9], as well as the thrombin-stimulated phosphoinositide turnover and cell proliferation, in hamster fibroblast (NIL) cells [10]. The thyrotropin-releasing hormone stimulation of polyphosphoinositide breakdown and prolactin release was shown to be effectively inhibited by neomycin in GH_3 rat pituitary cells [11]. Thus, neomycin, at low concentrations (0.05-1 mM), can be used as a probe to study the involvement of phosphoinositide metabolism in various cellular processes. In addition, other Ca^{2+}-dependent processes such as

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Abbreviations: [^{3}H]NA, tritiated noradrenaline; PIP_2, phosphatidylinositol diphosphate; IP_3, inositol 1,3,4-triphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; CNS, central nervous system

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histamine secretion from mast cells [12] and amylase secretion, stimulated by carbachol in permeabilized pancreatic acini [13], were shown to be inhibited by neomycin. PIP_2 bound to neomycin is not cleaved by phospholipase C to give IP_3 which mobilizes Ca^{2+} from internal stores, thus providing an explanation for neomycin's inhibitory action of Ca^{2+}-dependent processes. Furthermore, it has recently been shown that neomycin also binds directly to IP_3 [14], which can explain its direct inhibitory effects of cellular processes which utilize intracellular Ca^{2+}.

In the present study, we looked at the effect of neomycin on two Ca^{2+}-dependent release processes in the nervous system. We demonstrate that the stimulated release of [^{3}H]NA from cerebral cortical slices either by high potassium ions, or by veratridine is inhibited by neomycin, and suggests a role for phosphoinositide metabolism in the stimulated release process.

2. EXPERIMENTAL

2.1. *Chemicals*

[^{3}H]Noradrenaline (19.7 Ci/mmol) was purchased from New England Nuclear (USA). Neomycin sulfate and veratridine were from Sigma (USA). All other reagents and salts were of the highest purity available. All solutions were prepared using double distilled water.

2.2. *[^{3}H]NA release*

Male albino rats (150–200 g) were killed and their brains rapidly removed. Slices of cerebral cortex were prepared using a McIlwain tissue chopper (350 μm), and were allowed to equilibrate for 15 min with an oxygenated solution (95% O_2/5% CO_2) Krebs-Henseleit, containing the following (mM): 118 NaCl; 4.7 KCl; 1.2 $MgSO_4$; 25 $NaHCO_3$; 1.0 Na_2HPO_4; 0.004 Na_2EDTA; and 11.1 glucose, at 37°C. Subsequently, the slices were preincubated for 15 min with [^{3}H]NA 19.7 Ci/mmol, final concentration 0.25 μM, followed by 3 min washes with 3 ml of the oxygenated medium. The slices were distributed into superfusion chambers, containing 1 ml of the oxygenated solution, with an additional 1.3 mM $CaCl_2$. The spontaneous release of [^{3}H]NA was monitored during 60 min at 10 min intervals. At t = 60 min, the slices were stimulated either by 25 mM KCl (NaCl content in the medium was corrected accordingly) or 3 μM veratridine, for 10 min. Neomycin was added at t = 40 min, and was present in the assay throughout the experiment. At the end of the experiment, the remaining [^{3}H]NA content of tissue was extracted with 1 ml of 0.1 N HCl.

2.3. *Analysis of release assay*

[^{3}H]NA efflux during the 10 min collection period was expressed as the percentage of [^{3}H]NA content of the slices at the beginning of the collecting interval. The net efflux of [^{3}H]NA stimulated by either K^+ or veratridine was determined by subtracting the spontaneous efflux of radioactivity from the total overflow, which includes one fraction after stimulation in the case of K^+, and two fractions in the case of veratridine. In synaptosomes, release by K^+ was calculated in two fractions. Spontaneous [^{3}H]NA release was about 2–3% of total tissue content, and stimulated [^{3}H]NA release was up to 8–10%. Each experiment was carried out in quadruplicate determinations.

2.4. *Preparation of cortical synaptosomes*

Male albino rats (150–200 g) were killed by decapitation, and the brains were rapidly removed and dissected at 4°C. The cortex (0.5 g) was excised and homogenized (8 strokes) in 10 ml of 0.32 M sucrose, 4 μM EDTA, at 4°C, in a Teflon glass homogenizer (Hinkle & Kunkle). The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatant at $12\,000 \times g$ for 20 min. The pellet was collected and diluted to 20 ml final volume of Krebs-Henseliet buffer, containing (mM): 125 NaCl; 3 KCl; 1.2 $MgSO_4$; 1.2 $CaCl_2$; 1.2 $NaHPO_4$; 25 $NaHCO_3$; 11.1 glucose, and 4 μM EDTA. The synaptosomes were preincubated with oxygenated Krebs-Henseliet buffer, 95% O_2/5% CO_2 at pH 7.3, and loaded with 0.06 μM [^{3}H]NA (19.7 Ci/mmol) for 20 min, in the presence of 1 mM ATP and 1 μM GTP. Centrifugation at $600 \times g$ followed, and the synaptosomes were then diluted to a final concentration of 0.5 mg/ml.

2.5. *[^{3}H]NA release in cortical synaptosomes*

The preloaded synaptosomes (11.0 mg protein) were layered on Sephadex G-15 in a final volume of 1.5 ml and incubated in oxygenated buffer for 45 min. Protein was determined according to Lowry et al. [15] using bovine serum albumin as a

standard. The synaptosomes were stimulated with 15 mM KCl for 2 min and neomycin, at the indicated concentration, was added 8 min prior to the K^+ stimulation. Efflux of radioactivity was monitored at intervals of 2 min. At the end of the experiment, total residual [^{3}H]NA content was determined by extraction of the tissue with 0.1 N hydrochloric acid. Radioactivity in the withdrawn samples was determined in liquid scintillation counting with 50% efficiency. The induced [^{3}H]-NA release during the stimulation period was expressed as a percentage of total tritium present at the onset of stimulation.

3. RESULTS

3.1. *Stimulation of [^{3}H]NA release by K^+ (25 mM) in cerebral cortical slices*

Rat cerebral cortical slices prelabeled with [^{3}H]NA were allowed to reach a steady spontaneous flow of radioactivity, and at $t = 60$ min were exposed to 25 mM KCl for 10 min. An evoked release of [^{3}H]NA of 8.6 ± 0.4% of total tissue [^{3}H]NA, above spontaneous release, was observed (fig.1). The K^+ evoked release of [^{3}H]NA was absolutely dependent on the presence of Ca^{2+} in the medium (not shown).

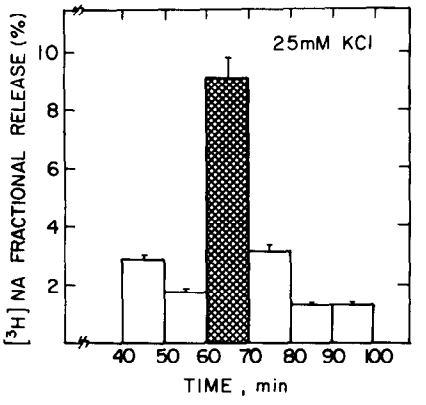

Fig.1. Potassium-stimulated [^{3}H]NA release. Rat cerebral cortical slices, prelabelled with [^{3}H]NA were incubated with oxygenated medium, and the radioactivity released during 10 min periods was collected. At $t = 60$ min, 25 mM KCl (osmolality of the medium was corrected by lowering NaCl content, respectively) was added to the incubation medium for 10 min (see hatched area). Fractional release was calculated as described in section 2. Each column represents the mean value of 4 independent determinations, and the bars depict SE.

3.2. *Stimulation of [^{3}H]NA release by veratridine in cerebral cortical slices*

Cortical slices, prelabeled with [^{3}H]NA were allowed to reach a steady spontaneous efflux of radioactivity, and at $t = 60$ min, were exposed to 3.0 μM veratridine for a period of 10 min. Veratridine, a veratrum alkaloid which depolarises excitable membranes, at 3.0 μM, caused a 13.4 ± 0.3% increase in [^{3}H]NA fractional efflux above spontaneous release. The results represent the mean of quadruplicate determinations carried out twice (fig.2). At 3 μM veratridine, [^{3}H]NA release was absolutely dependent on extracellular calcium (not shown).

3.3. *Stimulation of [^{3}H]NA release by 15 mM K^+ in rat brain synaptosomes*

Cortical synaptosomes prelabeled with [^{3}H]NA were superfused with medium (gassed with 95% O_2/5% CO_2) at 37°C for 45 min. KCl (15 mM) was added to the medium at $t = 55$ min for 2 min. The induced [^{3}H]NA release, collected at 2 min intervals (see section 2), was 3-fold of the control superfusion system run in parallel. The spontaneous

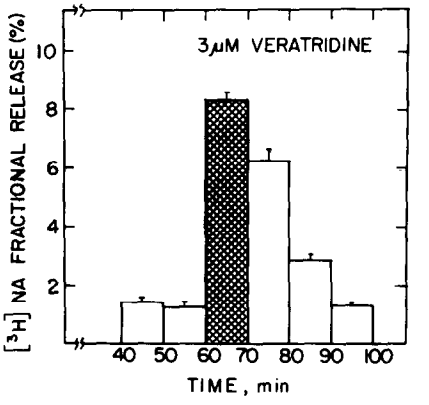

Fig.2. Veratridine stimulation of [^{3}H]NA release. Rat cerebral cortical slices prelabeled with [^{3}H]NA were incubated with oxygenated medium, and the radioactivity released during 10 min periods was collected. At $t = 60$ min, 3 μM veratridine was added to the incubation medium for a 10 min interval (see hatched area). Fractional release was calculated as described in section 2. Each column represents the mean value of 4 independent determinations, and the bars depict SE.

fractional [^{3}H]NA release was 2–2.5% of total [^{3}H]NA in control, as compared to 6–7.5% of total [^{3}H]NA in the stimulated system.

3.4. *The effect of neomycin on the K^+-stimulated [^{3}H]NA release in cortical slices*

Neomycin, at indicated concentrations (fig.3), was added to rat cerebral cortical slices at $t = 40$ min, and was present in the medium throughout the experiment. The spontaneous [^{3}H]NA fractional release during two 10-min intervals, was not affected by neomycin. At $t = 60$ min, the slices were stimulated by 25 mM K^+ (fig.1) for 10 min. The total K^+-induced [^{3}H]NA release was calculated by subtracting the spontaneous efflux of radioactivity from the total [^{3}H]NA released during the stimulation period and the following 10 min interval. As shown in fig.3, the net fractional release decreases as a function of neomycin concentration, with an apparent half-maximal effect, $IC_{50} = 0.5 \pm 0.07$ mM (fig.3). The results are the mean value of four independent release systems. In all cases the SE was $< 15\%$ of the mean.

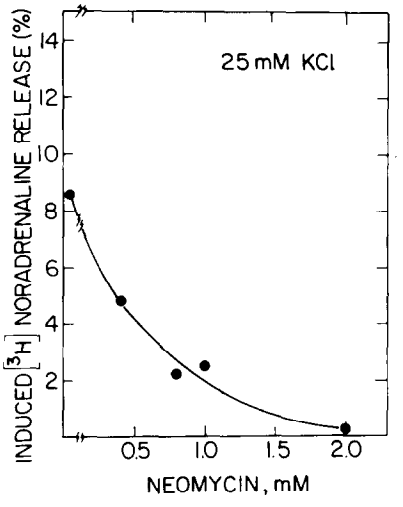

Fig.3. Effect of neomycin on 25 mM K^+-induced [^{3}H]NA release. Rat cortical slices labeled with [^{3}H]NA, were stimulated with 25 mM K^+ for 10 min at $t = 60$ min. Neomycin was added to the medium at $t = 40$ min, 20 min prior to stimulation. Data are expressed as total K^+-induced [^{3}H]NA released in the absence and presence of neomycin at concentrations as indicated. Total [^{3}H]NA release induced by K^+ was 8.5 ± 0.6% of total tissue content of [^{3}H]NA. Each point represents the mean ± SE of 4 independent determinations ($n = 4$).

3.5. *The effect of neomycin on the veratridine-induced [^{3}H]NA release in cortical slices*

Neomycin, at indicated concentrations, was added to rat cerebral cortical slices at $t = 40$ min. After two 10-min intervals, during which no effect on [^{3}H]NA spontaneous release was observed, at $t = 60$ min, the slices were stimulated by 3.0 μM veratridine. The total veratridine-induced [^{3}H]NA release was calculated by subtracting the spontaneous efflux of radioactivity from the total [^{3}H]NA released during the stimulation period and the following 20 min. As shown in fig.4, a decrease in the net stimulated [^{3}H]NA release was observed as a function of neomycin concentration, with an apparent half-maximal effect, $IC_{50} = 0.65 \pm 0.1$ mM. The result is the mean of four independent release systems. In all cases, the SE was $< 15\%$ of the mean.

3.6. *The effect of neomycin on the K^+-induced [^{3}H]NA release in synaptosomes*

As shown in fig.5, neomycin inhibits the K^+-stimulated release of [^{3}H]NA from cortical synap-

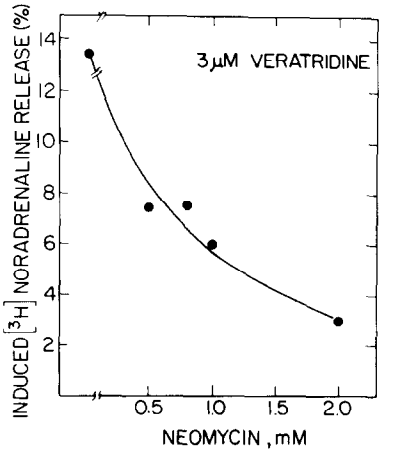

Fig.4. Effect of neomycin on varatridine-stimulated [^{3}H]NA release. Cortical slices, labeled with [^{3}H]NA, were stimulated with 3 μM veratridine at $t = 60$ min for 10 min. Neomycin was added to the medium (at the indicated concentrations) 20 min prior to stimulation ($t = 40$ min). Data are expressed as total veratridine-stimulated [^{3}H]NA released in the absence and presence of neomycin. Total [^{3}H]NA release induced by veratridine was 13.5 ± 0.5% of [^{3}H]NA tissue content. Each point represents the mean ± SE of 4 independent determinations ($n = 4$).

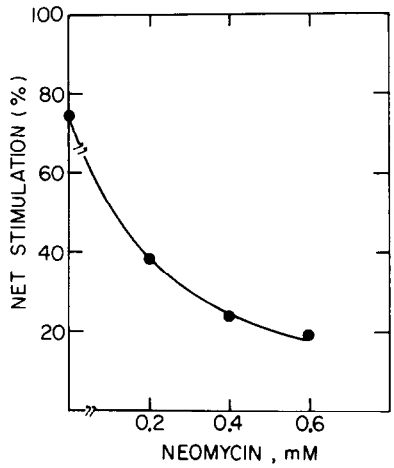

Fig.5. Effect of neomycin on K^+-induced [^{3}H]NA release in brain cortical synaptosomes. Synaptosomes prepared from rat cortex, prelabeled with [^{3}H]NA, were incubated with control oxygenated medium for 45 min. Stimulation was initiated by KCl (15 mM), at $t = 55$ min for 2 min. Neomycin at the indicated concentrations was added 8 min prior to stimulation. [^{3}H]NA release was calculated as the net [^{3}H]NA after subtracting the spontaneous release ($n = 4$). Each point represents 4 independent release systems ($n = 4$) and the results are the average ± SE of the net % stimulation.

tosomes, with an apparent half-maximal effect, $IC_{50} = 0.2 \pm 0.03$ mM.

4. DISCUSSION

The mechanism via which calcium ions trigger neurotransmitter release is still unknown, and one of the essential points raised is the source of the calcium ions needed in the process. Recent studies have correlated the neomycin inhibitory effect of various Ca^{2+}-dependent cellular processes with the availability of phosphatidylinositol 1,2-diphosphate for phospholipiase C [9–13].

In the present study, neomycin inhibits the K^+- and the veratridine-stimulated release of [^{3}H]NA, from either cerebral cortical slices or synaptosomes, at half-maximal values of 0.2–0.7 mM. The 50% inhibitory concentrations of neomycin are in the range of its affinity ($K_m = 0.53$ mM) as a competitive inhibitor of phospholipase C in the soluble fractions of rat renal tissue [16]. Both K^+ (25 mM) and veratridine (3 μM) stimulate neurotransmitter release as Ca^{2+}-dependent processes, and both were shown to increase the level of phosphoinositides [17], which is one of the routes for eliciting a rapid rise in intracellular calcium ions. Since stimulation of neurotransmitter release by K^+ and veratridine depends on the presence of extracellular Ca^{2+} as recently shown using Fura-2 [18], and in both processes Ca^{2+} entry is essential, the question arises as to the role of intracellular Ca^{2+} in this process.

Recently, Irvine and Moor [19] have provided indirect evidence to suggest that IP_4, which is formed by phosphorylation of IP_3, controls the entry of extracellular calcium ions. They show that injection of IP_3 into fertilized eggs leads to both an increase of intracellular Ca^{2+} and stimulation of Ca^{2+} influx across the cell membrane [19]. Thus, the inhibitory effect of neomycin, which selectively binds anionic phosphoinositides [1–6] as well as IP_3 [14], can originate from lowering the amount of IP_3 which consequently leads to a decrease in the amount of IP_4. Therefore, neomycin is expected to inhibit release processes which are dependent on Ca^{2+} influx across the cellular membrane as well as processes which involve Ca^{2+} mobilization from internal stores.

Further support for this hypothesis can be derived from the report by Baird and Nahorski [20], who observed a rapid rise in IP_4 formation, followed by K^+ stimulation of cortical rat brain slices. It is therefore essential to demonstrate that neomycin is able to lower IP_3 and IP_4 levels formed concomitant to the induction of neurotransmitter release by either K^+ or veratridine. These experiments are now in progress.

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