Br. J. Pharmac. (1979), **65**, 271–276

RELEASE OF [^{3}H]-ACETYLCHOLINE FROM THE ISOLATED RETINA OF THE RAT BY POTASSIUM DEPOLARIZATION: DEPENDENCE ON HIGH AFFINITY CHOLINE UPTAKE

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1 The effect of potassium depolarization on the release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from the isolated retina of the rat was studied.

2 Exposure of retinae to medium containing KCl (50 mM) evoked a large increase in the efflux of [^{3}H]-ACh with only a small concurrent increase in the efflux of [^{3}H]-choline. The KCl-evoked release of [^{3}H]-ACh was almost abolished in calcium-free medium.

3 Incubation of retinae with [^{3}H]-choline in sodium-free medium, or medium containing hemicholinium-3 (HC-3), procedures that are believed to inhibit selectively the high affinity choline transport system, reduced the retinal uptake of [^{3}H]-choline by approximately 50% and the synthesis of [^{3}H]-ACh by about 97%.

4 The potassium-evoked release of [^{3}H]-ACh was almost abolished in retinae that had been loaded with [^{3}H]-choline in sodium-free medium or medium containing HC-3, and subsequently superfused in normal medium.

5 It is suggested that as in other areas of the nervous system, a sodium-dependent, high affinity uptake system for choline is important in retinal cholinergic nerve terminals.

Introduction

The retina possesses both high and low affinity uptake processes for choline, and as in other areas of the central and peripheral nervous systems, the retinal high affinity uptake system is sodium-dependent and associated with a high degree of acetylcholine (ACh) formation (Neal & Gilroy, 1976). Choline uptake systems with these properties are believed to be uniquely localized in cholinergic nerve terminals (Kuhar, Sethy, Roth & Aghajanian, 1973; Kuhar, Dehaven, Yamamura, Rommelspacher & Simon, 1975; Suszkiw & Pilar, 1976; Higgins & Neal, 1977; 1978; Kuhar & Murrin, 1978).

In the present study, we have examined the effect of potassium depolarization on the release from the retina of [^{3}H]-ACh newly synthesized from [^{3}H]-choline. By varying the loading conditions, we have attempted to establish the role of the high affinity choline uptake system in the potassium-evoked release of ACh from the retina.

Methods

Incubation and superfusion of tissue

Wistar rats were killed by cervical dislocation and their eyes enucleated. The retinae were dissected and pairs of retinae (approximate weight 20 mg) were given a preliminary incubation for 15 min in 5 ml of Krebs bicarbonate Ringer at 37°C. The incubation flasks were gassed with 5% CO_2 in O_2. [^{3}H]-choline (sp. act. 13 Ci/mmol; Radiochemical Centre, Amersham) was then added to the medium to give a final concentration of 0.2 μM and the incubations were continued for 30 min. The retinae were then removed and washed in fresh medium. For each superfusion, two retinae were placed between pieces of nylon mesh in a small chamber (volume = 1 ml) and superfused at a rate of 1.2 ml/min with freshly gassed medium. Two min samples were collected automatically in

0007-1188/79/020271-06 $01.00

counting vials, and after the addition of Aquasol (New England Nuclear) (10 ml), the radioactivity in each sample was measured by liquid scintillation counting. The counts obtained were converted to d/min by the external standard ratio method.

Separation of [^3H]-acetylcholine and [^3H]-choline in superfusates

This was undertaken in preliminary experiments to establish the identity of the labelled compounds released from the retina by potassium depolarization. In these experiments, physostigmine (30 μM) was included in the superfusion medium and 4 min fractions (4.8 ml) were collected into siliconized tubes containing 1 M HCl (100 μl). This procedure ensured the preservation of [^{3}H]-ACh in the samples since the pH was immediately adjusted to approximately 4. [^{14}C]-choline was added to each sample as an internal standard and they were then freeze dried. Acetonitrile (500 μl), acidified with glacial acetic acid (1%), was added to each tube and the residues were dispersed with a glass rod. The tubes were then immersed in a water bath at 85°C for 1 min to dissolve the choline salts. The remaining residues were spun down by centrifugation and the supernatants removed. These were evaporated to dryness in a stream of air and the residues were dissolved in 95% ethanol (50 μl) acidified to pH 4 with acetic acid. The samples (50 μl) were then subjected to high voltage paper electrophoresis to separate choline and acetylcholine (Potter & Murphy, 1967). The spots were visualized with iodine and the radioactivity measured by double label liquid scintillation counting. The final recovery was estimated by the recovery of the [^{14}C]-internal standard which also indicated the overlap from choline to ACh (Feigenson & Saelens, 1969). The overlap was less than 0.5%.

Results

Uptake and metabolism of [^3H]-choline

Retinae were incubated with [^{3}H]-choline (0.2 μM) for 30 min at 37°C. At this concentration, calculations using previously determined kinetic constants indicated that approximately 50% of the choline was transported by the high affinity process. Under these conditions, tissue: medium ratios of 3.7 ± 0.30 (mean ± s.e mean of 9 determinations) were obtained. In 6 experiments, the retinae incubated with [^{3}H]-choline were then washed in fresh medium and homogenized in 0.15 M formic acid in acetone. The homogenates were centrifuged and ^{3}H-metabolites in the supernatant were separated by high voltage electrophoresis and determined as described previously. Of the radioactivity recovered from the retinae, 19.9 ± 1.98% was present as [^{3}H]-ACh; 30.2 ± 3.92% as [^{3}H]-choline, the remainder occurring as ^{3}H-phosphorylated metabolites. The recovery of radioactivity was between 83 and 90%.

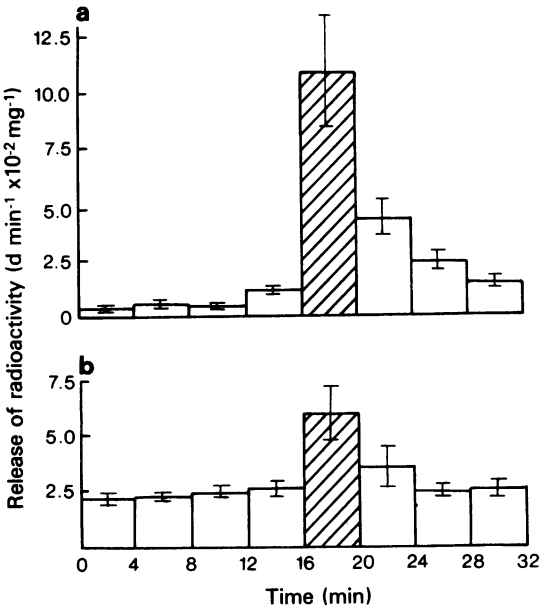

Figure 1 Effect of KCl (50 mM, hatched column) on the efflux of [^{3}H]-acetylcholine (a) and [^{3}H]-choline (b) from rat retinae incubated with [^{3}H]-choline and then superfused with medium containing physostigmine (30 μM). Each result is the mean of 6 experiments; vertical lines show s.e. mean.

Incubation of retinae in either sodium-free medium or medium containing hemicholinium-3 (HC-3, 100 μM) (6 determinations in each case) only reduced the uptake of radioactivity to 58 ± 4.2% and 49.5 ± 7.21% of control values respectively but reduced [^{3}H]-ACh synthesis to 2.1% and 1.6% of control respectively. The finding that the uptake of [^{3}H]-choline was inhibited by about 50% but [^{3}H]-ACh synthesis was almost abolished in sodium-free medium or in medium containing HC-3 suggests that incubations under these conditions selectively inhibit the retinal high affinity choline transport system.

Effect of potassium depolarization on [^3H]-acetylcholine release

The release of [^{3}H]-ACh and [^{3}H]-choline from the retina is illustrated in Figure 1. The spontaneous resting release of radioactivity was mainly [^{3}H]-choline, only a small proportion (18.1 ± 2.91% mean ± s.e. mean of 6 experiments) being [^{3}H]-ACh. However, potassium depolarization caused a striking increase

in [^{3}H]-ACh release (21.8 ± 5.73 times the resting release) but only a small increase in [^{3}H]-choline efflux (2.1 ± 0.27 times the resting release). Thus, at the peak of the potassium-evoked response, 65% of the radioactivity was present as [^{3}H]-ACh. These results indicate that at least 75% of all the radioactivity released in response to potassium depolarization was [^{3}H]-ACh. Thus, in subsequent experiments, physostigmine was omitted from the medium, and the radioactivity released by potassium stimulation was taken as a measure of [^{3}H]-ACh release.

Effect of calcium on potassium evoked release of [^{3}H]-acetylcholine

The spontaneous resting release of radioactivity from the retina gradually declined over the first 30 min of superfusion and then remained steady. The fractional rate coefficient (f) at this stage being in the range 0.0005 to 0.0015/min. (Use of the fractional rate coefficient in these experiments was for operational convenience only and does not imply knowledge of the [^{3}H]-ACh pool).

Exposure of retinae to KCl (50 mM) for 2 min rapidly increased the efflux of radioactivity to 3.84 times the resting release (Table 1). This potassium-evoked release of radioactivity from the retina was almost abolished when the tissue was superfused with calcium-free medium and under these conditions the potassium evoked release was only 1.6 times the resting release. The results are summarized in Table 1 and a typical experiment is illustrated in Figure 2.

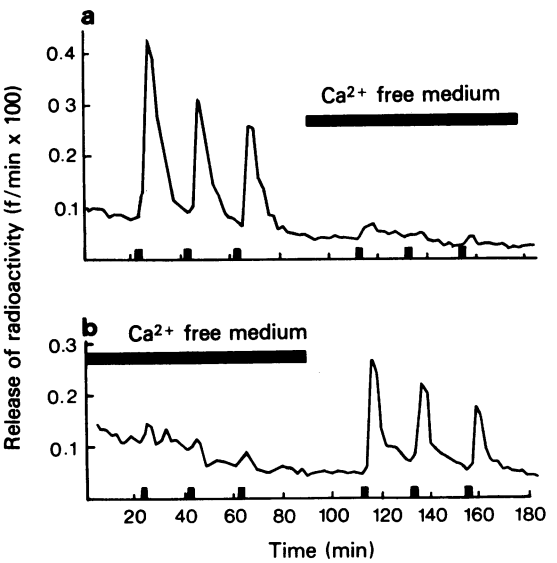

Figure 2 Effect of $[Ca^{2+}]_o$ on the potassium-evoked release of [^{3}H]-acetylcholine from the retina. In these experiments two superfusions were performed simultaneously and the results obtained in one pair of superfusions are illustrated (a and b). ■ = exposure of retina for 2 min to medium containing KCl (50 mM). In this and subsequent figures the efflux of radioactivity is expressed as the fractional rate constant (f/min).

Effect of incubation in sodium-free medium

Retinae were incubated with [^{3}H]-choline (0.2 μM) in 50 mM Tris/HCl buffered medium (pH = 7.4) in which the sodium ions were replaced with sucrose. Under these conditions the total uptake of [^{3}H]-choline was reduced by approximately 40% compared with control retinae which were incubated in Tris buffered medium containing the normal concentration of sodium. The retinae were washed in sodium-free medium and then superfused with unmodified Krebs bicarbonate Ringer.

When retinae were loaded with [^{3}H]-choline in sodium-free incubation medium the effect of potas-

Table 1 Effect of different incubation conditions on the potassium evoked release of [^{3}H]-acetylcholine from the retina of the rat

Incubation conditions	*Relative efflux coefficient (f min^{-1} peak evoked release/f min^{-1} spont. resting release)*		
	Normal superfusion medium	*0[Ca^{2+}]$_o$ superfusion medium*	P
Krebs bicarbonate Ringer (controls)	3.84 ± 0.03	1.60 ± 0.06	<0.001
Sodium-free medium	1.43 ± 0.03	1.28 ± 0.03	NS
Hemicholinium-3 (100 μM)	1.70 ± 0.03	1.47 ± 0.05	<0.02

Retinae were loaded with [^{3}H]-choline in Krebs bicarbonate Ringer (controls), in sodium-free medium, or in medium containing hemicholinium-3. In all cases, the tissues were subsequently superfused with both unmodified and calcium-free Krebs bicarbonate Ringer solution and the effects of potassium depolarization were studied. Each result is the mean ± s.e. mean of 6 experiments. The result from each experiment was itself the mean of 3 responses to KCl (50 mM).

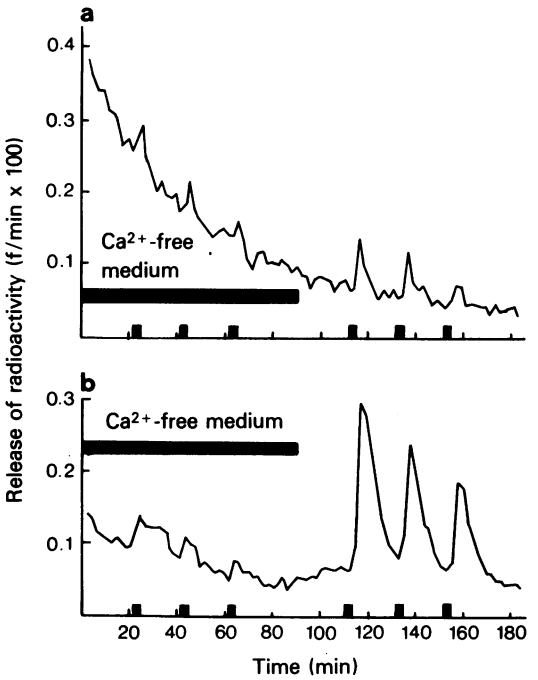

Figure 3 Effect on the calcium-dependent and independent potassium-evoked release of [^{3}H]-acetylcholine from retina loaded with [^{3}H]-choline in sodium-free medium (a) or medium containing normal sodium (b, control). ■ = KCl (50 mM).

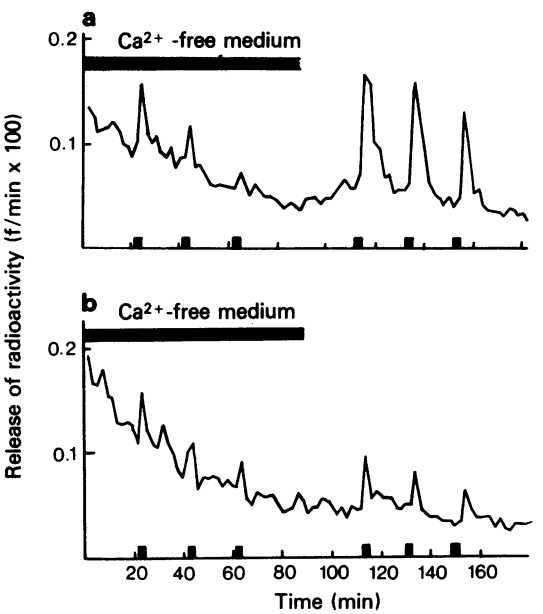

Figure 4 Effects on the calcium-dependent and independent potassium-evoked release of [^{3}H]-acetylcholine from retinae loaded in the presence (b) or absence (a) of hemicholinium-3 (100 μM).

sium depolarization on release of radioactivity was almost abolished (Figure 3). Thus, potassium increased the efflux of radioactivity by only 1.43 times the resting release (Table 1). This small increase in radioactivity release was not significantly different from that obtained in control experiments in the absence of calcium (Table 1). Furthermore, the potassium-evoked, calcium independent release of radioactivity was not significantly affected by loading the tissue in sodium-free medium (Table 1).

Effect of incubation with hemicholinium

Retinae were incubated with [^{3}H]-choline (0.2 μM) in the absence (controls) or presence of HC-3 (100 μM). This concentration of HC-3 reduced the total uptake of [^{3}H]-choline by approximately 50%. The retinae were then superfused in HC-3-free medium.

Incubation of retinae with [^{3}H]-choline in the presence of HC-3 greatly reduced the potassium-evoked release of radioactivity in the subsequent superfusion (Figure 4). Thus, the potassium-evoked release was 1.7 times the resting release (Table 1); this potassium-evoked release with HC-3 was further reduced when calcium was omitted from the superfusion medium (Table 1).

Discussion

In the present study we have found that potassium depolarization evokes a calcium-dependent release of [^{3}H]-ACh from the rat retina *in vitro*. This confirms previous reports in which potassium has been shown to increase the release of radioactivity or [^{3}H]-ACh from rat or chicken retinae loaded with [^{3}H]-choline *in vitro* (Neal, 1976a; Baughman & Bader, 1977).

The potassium-evoked release of [^{3}H]-ACh from the retina appears to depend on a high affinity uptake process for choline because it was abolished when retinae were loaded with [^{3}H]-choline under conditions believed to inhibit selectively the high affinity uptake system. Thus, when retinae were incubated with [^{3}H]-choline in sodium-free medium, the uptake was reduced by 40% but the potassium-evoked release of [^{3}H]-ACh was almost abolished. Since high affinity choline uptake processes are usually highly sodium-dependent, whilst low affinity processes show little if any sodium-dependence (see Kuhar & Murrin, 1978 for refs.), these results strongly suggest that the [^{3}H]-ACh release by potassium depolarization is associated with the high affinity

uptake process. Furthermore, this process is probably located in cholinergic nerve terminals since absence of sodium ions in the medium during incubation with [^{3}H]-choline reduced the synthesis of [^{3}H]-ACh to less than 3% of that found in controls.

This conclusion is supported by experiments with HC-3, which in synaptosomes has been shown to inhibit both high and low affinity choline uptake systems with *Ki* values of 1.1 μM and 122 μM respectively (Yamamura & Snyder, 1973). In the present experiments, HC-3 at a concentration of 100 μM inhibited [^{3}H]-choline uptake in the retina by 50% and almost abolished the synthesis of [^{3}H]-ACh. These results are very similar to those obtained in sodium-free medium, suggesting that HC-3 at a concentration of 100 μM, inhibits mainly the high affinity choline uptake system. The potassium-evoked release of [^{3}H]-ACh from retinae loaded with [^{3}H]-choline in the presence of HC-3 (100 μM) was almost abolished, again supporting the view that the high affinity uptake process is essential in providing a source of [^{3}H]-choline for the synthesis of [^{3}H]-ACh in retinal cholinergic nerve terminals.

The present results are in close agreement with those obtained from similar experiments with hippocampal slices (Mulder, Yamamura, Kuhar & Snyder, 1974) and provide evidence for the importance of a sodium-dependent, high affinity, choline uptake process in the cholinergic system of the retina.

The cellular sites of uptake of [^{3}H]-choline in the rat retina are unknown, but in the chicken retina, autoradiographical studies indicate that [^{3}H]-choline is selectively accumulated by some amacrine cells and a small proportion of bipolar cells (Baughman & Bader, 1977). This is consistent with other evidence which suggests that cholinergic synapses in the retina occur predominantly in the inner plexiform layer and that a sub-population of amacrine cells is cholinergic (Nichols & Koelle, 1968; Neal, 1976a; Ross & McDougal, 1976; Masland & Ames, 1976; Vogel, Maloney, Ling & Daniels, 1977; Massey & Neal, 1978). If the sites of [^{3}H]-choline uptake in the rat retina are similar to those in the chicken (but see Neal (1976b) for the importance of species differences in the retina) then the [^{3}H]-ACh released by potassium depolarization presumably originated from amacrine and/or bipolar cells.

Potassium is able to release transmitter substances, such as γ-aminobutyric acid (GABA), from glia as well as neurones (Bowery & Neal, 1978). However, it seems unlikely that [^{3}H]-ACh released from the retina originates from the Müller cells because (1) there is little evidence that these glial cells possess significant choline acetyltransferase activity (Ross & McDougal, 1976); (2) the release of [^{3}H]-ACh is highly calcium-dependent whilst 'transmitter' release from glia is not (Bowery & Neal, 1978; Neal & Bowery, 1979) and (3) a calcium-dependent release of [^{3}H]-ACh from the rabbit retina both *in vitro* (Masland & Livingstone, 1976) and *in vivo* (Massey & Neal, 1978) in response to physiological stimulation with flashes of light has also been demonstrated.

S.C.M. is an S.R.C. scholar. We are grateful to the Smith, Kline & French Foundation for a grant to M.J.N.

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(Received June 16, 1978.)