Subcellular Origin of Cholinergic Transmitter Release from Mouse Brain

Abstract. *Samples of minced mouse forebrain were treated in a way that resulted in a high ratio of false cholinergic transmitter (acetylhomocholine) to true transmitter (acetylcholine) in a synaptic vesicle fraction, and a low ratio of false to true transmitter in the nerve terminal cytoplasm. The spontaneous release of cholinergic transmitters from this minced tissue occurred independently of calcium and had a ratio of false to true transmitter similar to that of the cytoplasm, whereas the evoked transmitter release required calcium and had a ratio of false to true transmitter similar to that of the vesicular fraction.*

The neurotransmitter acetylcholine (ACh) is stored in at least two subcellular pools within central cholinergic nerve endings: the cytoplasm and synaptic vesicles (*1, 2*). According to the classical model of a cholinergic nerve ending, ACh is synthesized in the cytoplasm and transported into vesicles, where it is released (*3*). Not all studies support this model, however. Some indicate that ACh is released from the nerve terminal cytoplasm (*4*), whereas others indicate that it is released from the cytoplasm and synaptic vesicles (*5*).

One means of ascertaining whether cholinergic transmitter is released from a cytoplasmic (S_3) or vesicular (P_3) pool upon stimulation is to treat cholinergic tissue in such a way that the contents of these two pools can clearly be distinguished before release. This is accomplished by incubating minced mouse forebrain in a Krebs solution containing a high concentration of potassium and lithium instead of sodium; this treatment depletes the P_3 fraction of its ACh content independently of the S_3 fraction (*6*). Upon subsequent exposure to normal Krebs solution containing ^{14}C-labeled homocholine, an analog of choline, the ratio of newly synthesized [^{14}C]acetylhomocholine ([^{14}C]AHCh) to ACh is, indeed, substantially higher in the P_3 fraction (7.3) than in the S_3 fraction (0.44) (*7*). We measured transmitter release from tissue treated as described above in order to test whether released transmitter had a ratio of false to true transmitter similar to that contained in the S_3 or the P_3 fraction.

Male albino mice (CD-1) were killed by cervical dislocation in a cold room (4°C) where the brains (minus cerebellum, pons, and medulla) were removed and sectioned through the median sagittal fissure and then placed in several hundred milliliters of ice-cold incubation medium. Each half was removed from the washing medium, weighed, minced, and maintained on a petri dish until the onset of incubation. Minced tissues were incubated for 30 minutes in 10 ml of Krebs solution with high potassium (32.7 m*M*) and lithium (117 m*M*) instead of sodium at 37°C in an atmosphere of 95 percent O_2 and 5 percent CO_2. After this incubation, the minced tissues were washed twice with 5 ml of ice-cold Krebs solution and incubated for another 30 minutes in Krebs bicarbonate solution (normal Krebs) containing [^{14}C]homocholine (0.1 m*M*; specific activity, 16.4 dpm/pmole) and paraoxon (3 μM). After the second incubation, the minced tissues were washed twice with 5 ml of ice-cold normal Krebs solution and then incubated a third time for 5 minutes in 3 ml of one of the following solutions, all of which contained paraoxon (3 μM): (i) Krebs, (ii) 35 m*M* K^+-Krebs, (iii) 35 m*M* K^+-Krebs with no added calcium plus 0.1 m*M* EGTA [ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid], (iv) 35 m*M* K^+-Krebs with 16 m*M* Mg^{2+}, (v) Krebs with no added calcium plus 0.1 mm EGTA, or (vi) Krebs with 16 m*M* Mg^{2+}. In a different set of experiments, minced forebrain was first incubated in normal Krebs solution, then in Krebs solution plus [^{14}C]homocholine (0.1 m*M*) and paraoxon (3 μM), and finally for 5 minutes in 3 ml of either normal Krebs or 35 m*M* K^+-Krebs solution. At the end of the third incubation, the samples were chilled and centrifuged, and a portion of the supernatant was used for determinations of [^{14}C]AHCh and ACh (*8*).

In transmitter released spontaneously, the ratio of [^{14}C]AHCh to ACh was 0.56 ± 0.09 (Table 1), clearly closer to the ratio determined for the S_3 fraction [0.44 ± 0.06, N = 12 (*7*)] than that for the P_3 fraction [7.3 ± 3.2, N = 12 (*7*)], suggesting that spontaneous release oc-

Table 1. Spontaneous and potassium-induced release of cholinergic transmitters from mouse brain. Minced tissues prepared from mouse forebrains were incubated for 30 minutes in a lithium solution (32.7 m*M* K^+ and Li^+ instead of Na^+) and incubated for another 30 minutes in Krebs solution with [^{14}C]homocholine and paraoxon to refill the vesicle-bound fraction with newly synthesized [^{14}C]AHCh and achieve a high ratio of [^{14}C]AHCh to ACh in the vesicle-bound fraction and a low ratio in the cytoplasmic fraction. The minced tissues were incubated a third time for 5 minutes in Krebs or 35 m*M* K^+-Krebs and paraoxon, and the amounts of both transmitters released into the respective media were determined and compared. Values are means ± standard errors (S.E.) for the number of brains in parentheses. The ratio of [^{14}C]AHCh to ACh represents the release of [^{14}C]AHCh relative to ACh induced by the incubation solution.

Third incubation medium	[^{14}C]AHCh (nmole/g wet weight)	ACh (nmole/g wet weight)	[^{14}C]AHCh/ACh
Krebs	2.0 ± 0.2 (24)	4.1 ± 0.7 (24)	0.56 ± 0.09 (24)
35 m*M* K^+-Krebs	6.0 ± 0.4 (40)*	4.6 ± 0.6 (40)†	8.1 ± 0.8 (39)*

*Significantly different at $P < .001$ from the value obtained for the normal Krebs incubation (unpaired Student's *t*-test). †Not significantly different at $P = .05$ from the value obtained for the normal Krebs incubation (unpaired Student's *t*-test).

Table 2. Effect of Ca^{2+} omission or Mg^{2+} elevation on the potassium-induced release of cholinergic transmitter from mouse brain. After incubation in lithium solution and then in Krebs solution with [^{14}C]homocholine and paraoxon, minced tissues were incubated a third time for 5 minutes in 35 m*M* K^+-Krebs, 35 m*M* K^+-Krebs with 16 m*M* Mg^{2+} or 35 m*M* K^+-Krebs with no added Ca^{2+} and 0.1 m*M* EGTA. Amounts of cholinergic transmitters released into the media are given as means ± S.E. for the number of brains in parentheses.

Third incubation medium	[^{14}C]AHCh (nmole/g wet weight)	ACh (nmole/g wet weight)
35 m*M* K^+-Krebs	5.0 ± 0.4 (12)	4.8 ± 0.6 (12)
35 m*M* K^+-Krebs (16 m*M* Mg^{2+})	3.1 ± 0.3 (12)*	6.0 ± 1.3 (12)†
35 m*M* K^+-Krebs	5.4 ± 0.5 (18)	3.0 ± 0.4 (18)
35 m*M* K^+-Krebs (no added Ca^{2+} and 0.1 m*M* EGTA)	3.8 ± 0.4 (18)‡	2.6 ± 0.6 (18)†

*Significantly different at $P < .005$ from the value obtained for 35 m*M* K^+-Krebs incubation (analysis of variance, 3 by 2 factorial for days and treatments). †Not significantly different at $P = .05$ from the value obtained for the 35 m*M* K^+-Krebs incubation (analysis of variance). ‡Significantly different at $P < .025$ from the value obtained for the 35 m*M* K^+-Krebs incubation (analysis of variance).

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curs from a cytoplasmic transmitter store rather than from a vesicle-bound store. In contrast, the extra release of transmitters induced by stimulation with K^+ (4.0 nmole of [^{14}C]AHCh and 0.5 nmole of ACh) had a ratio of 8.1 ± 0.8 (Table 1), close to that of the P_3 fraction and clearly different from that of the S_3 fraction; this result strongly suggests that evoked release originates from vesicle stores and not from cytoplasmic stores. Either omission of Ca^{2+} or elevation of Mg^{2+} reduced the evoked release of [^{14}C]AHCh, but not of ACh (Table 2). Neither treatment affected the spontaneous release of [^{14}C]AHCh [2.7 ± 0.1 versus 2.4 ± 0.1 nmole/g per 5 minutes (N = 8), and 2.2 ± 0.3 versus 1.6 ± 0.2 nmole/g per 5 minutes (N = 13), respectively] or of ACh (data not shown), a result which also suggests that the spontaneous release of cholinergic transmitter occurs from a different subcellular pool than the evoked release.

Treatment of brain tissue in lithium–high potassium Krebs solution, as compared to treatment of brain tissue in normal Krebs solution, increases the ratio of [^{14}C]AHCh to ACh markedly in the P_3 (0.46 to 7.3) and increases this ratio to a similar extent (0.28 to 8.1) when transmitter release is stimulated by K^+, without appreciably increasing the ratio in the S_3 (0.26 to 0.44). This result provides additional evidence that the K^+-induced release of cholinergic transmitter occurs from the vesicle-bound fraction.

We used a lithium–high potassium Krebs treatment of mouse brain tissue and homocholine to distinguish the transmitter content of the cytoplasmic and vesicle-bound fractions before release. This treatment selectively lowers the ACh content of the vesicle-bound fraction, possibly by depolarizing the tissue and releasing vesicle-bound ACh while simultaneously blocking the transport of extracellular precursor essential for refilling this fraction with acetylated product. Subsequent incubation of the treated tissue in normal Krebs with either [^{14}C]choline or [^{14}C]homocholine augments accumulation of the precursors by the P_3 fraction, independently of the S_3 fraction (*7*). The precursors may then be acetylated by choline *O*-acetyltransferase (E.C. 2.3.1.6) associated with this fraction (*9*, *10*) to achieve a ratio of [^{14}C]ACh to ACh or of [^{14}C]AHCh to ACh that is higher in the P_3 fraction than in the S_3 fraction (*7*). Homocholine is similar to choline in most respects [transport, acetylation by intact tissue, and release as an acetylated product (*11*, *12*)], but differs from choline in one very important respect. It is not acetylated by soluble choline acetyltransferase (*9*, *12*, *13*), believed to exist in the cytoplasm (*14*). Therefore, the high ratio of [^{14}C]AHCh to ACh achieved in the P_3 fraction during repletion may be due to its exclusive acetylation by membrane-bound choline acetyltransferase. The relatively low ratio of [^{14}C]AHCh to ACh that is simultaneously achieved in the S_3 fraction may be due to the inability of soluble choline acetyltransferase in the cytoplasm to acetylate homocholine.

This study confirms the predictions made by others (*5*) that the spontaneous release of cholinergic transmitter occurs from the cytoplasm independently of extracellular Ca^{2+}, whereas the evoked release of cholinergic transmitter occurs from the vesicle-bound fraction by a Ca^{2+}-dependent process.

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8. A 100-μl portion of the release medium was mixed with 300 μl of tetraphenylboron–3 heptatanone and centrifuged. Then 200 μl of the organic phase was transferred to a tube containing 100 μl of 1*N* HCl. After thorough mixing and centrifugation, the organic layer was discarded, and a 20-μl portion of the 1*N* HCl was transferred to a new tube and dried. The total amount of acetylated product ([^{14}C]AHCh + ACh) was determined [A. M. Goldberg and R. E. McCaman, *J. Neurochem.* **20**, 1 (1973)]. The amount of [^{14}C]AHCh was determined following the extraction described and drying of another 20-μl portion of 1*N* HCl in a tube. The labeled homocholine was converted to labeled phosphoryl homocholine with choline kinase (E.C. 2.7.1.32) and separated from acetylhomocholine with tetraphenylboron–3 heptanone (75 mg/ml). This procedure phosphorylates [^{14}C]homocholine standards linearly from 25 through 200 pmole, and virtually none of the phosphorylated homocholine is extracted into the organic phase. The extraction of [^{14}C]AHCh from the aqueous phase into the organic phase is complete. The amount of ACh present in the release media was estimated by subtracting the amount of [^{14}C]AHCh from the total amount of acetylated product ([^{14}C]AHCh and ACh) measured by the Goldberg and McCaman assay done on each individual brain. The ACh standards were simultaneously extracted from each incubation medium used; the extraction efficiency was at least 70 percent for all media used. The efficiency of [^{14}C]AHCh extraction from these media was also determined for each experiment.
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Oral Contraceptives, Lanosterol, and Platelet Hyperactivity in Rat

Abstract. *Lanosterol, a cholesterol precursor that increases considerably in the platelets of rats treated with oral contraceptives, was incubated with either platelet-rich plasma or washed platelet suspension. After 2 minutes there was a remarkable dose-related increase in platelet activity. This platelet hyperactivity as measured by clotting time and platelet aggregation could not be reproduced by cholesterol or ethinylestradiol.*

An increase in the activity of several plasma factors has been noted in women taking oral contraceptives (*1*) but, apparently, the mechanism of this enhanced activity has not been elucidated. It is even a matter of controversy whether the level of these plasma factors has clinical significance (*2*). Our group has shown (*3*, *4*) that the hypercoagulability resulting from oral contraceptives might depend essentially on an increase in the clotting activity of platelets.

There are also conflicting reports concerning the effect of oral contraceptives

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