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INCREASED CATECHOLAMINE RELEASE FROM ADRENAL MEDULLA BY LIPOSOMES LOADED WITH SODIUM OR CALCIUM IONS*

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Abstract—Rat adrenal glands were incubated *in vitro* in calcium-free Locke's solution with 2 mM EGTA. Catecholamine release from adrenals was unaffected by addition of liposomes loaded with K^+. Addition of Ca^{2+}-loaded liposomes or Na^+-loaded liposomes induced a substantial increase of CA release. When liposomes were preloaded with Na^+ and EGTA the CA-releasing effect was abolished.

Catecholamines (CA) can be released from adrenal medulla by various stimuli, e.g. acetylcholine (the natural transmitter, which is secreted by pre-ganglionic nerve endings) or increased potassium concentration in the medium (causing depolarization) [1]. Incubation of adrenals *in vitro* in a potassium-free medium or in the presence of ouabain (10^{-3} M) also induces increased CA secretion [2]. The latter stimuli are known to cause inactivation of the sodium pump. This suggests that the increased CA secretion may be ascribed to inhibition of the sodium pump (which results in an increase of the intracellular concentration of sodium, $[Na^+]_{in}$). Release of CA is an exocytotic process, mediated by calcium [1]. It may, therefore, be inferred that increased $[Na^+]_{in}$ causes an increase of intracellular calcium concentration ($[Ca^{2+}]_{in}$). The ability of intracellular sodium to release calcium ions from intracellular stores has been suggested in several systems [3–5]. However, no direct information exists for such a process in the adrenal medulla.

One way to test the validity of this hypothesis would be to introduce the appropriate ions directly into the adrenal medullary cells. Introduction into cell cytoplasm of molecules which can not pentrate the cell membrane is possible through the use of small unilamellar vesicles (SUV, liposomes). These spherical membranes can be obtained by ultrasonic irradiation of aqueous dispersions of various phospholipids [6]. In the course of this process, solutes are entrapped within the aqueous phase of the liposomes. Liposomes interact with cell membranes, so that the entrapped solutes are introduced into the cells. This has been shown in both tissue cultures [7–10] and in intact animals [11, 12]. Endocytosis [13, 14] and liposome–cell fusion [8, 15] have been proposed as alternate mechanisms for this interaction. Fusion is favoured when the experiments are carried out at temperatures higher than the gel–liquid transition temperature of the lipids constituting the liposomes [7, 16].

In the present study we used vesicles prepared from egg phosphatidylcholine (PC). The phase-transition temperature of this phospholipid is much lower than room temperature. Cholesterol was also introduced into the liposomes, so as to lower ion permeability [17, 18] and ensure that the fusion would result in smaller changes in the physical properties of the cholesterol-containing cell membranes [17]. In some experiments, dicetylphosphate (DCP) was also added, to increase the rate of vesicle–cell association [15].

In order to eliminate the possibility of calcium being introduced into the adrenal medullary cells from the incubation medium, rather than through cell–vesicle interactions, all incubation media were calcium-free and contained 2 mM EGTA.

MATERIALS AND METHODS

Incubation. Rat adrenals were obtained immediately after cervical dislocation. The glands were cleaned and each adrenal was cut into two halves and placed in 50 ml Erlenmeyer flasks, in a thermostatic bath (37°) with constant shaking, as previously described [19]. The incubation medium consisted of 10 ml of calcium-free Locke's solution containing EGTA (2 mM). The incubation media included liposomes, containing different ions, as specified for each experiment. Incubation was carried out for 20 min. The glands were then separated from the medium.

Catecholamine assay. $HClO_4$ was added to the adrenals and to the incubation medium (final concentration 0.4 M). After extraction the CA were adsorbed on alumina columns, as previously described [20]. The trihydroxyindole method was used for assay of CA, in an Aminco–Bowman spectrofluorimeter [20]. Results are expressed as μg CA released per gland and as per cent of initial total CA content released during incubation.

Preparation of liposomes. Liposomes were prepared from mixtures of chromatographically pure egg phosphatidylcholine (Makor Chemicals, Jerusalem), cholesterol (Sigma), and, in some instances, dicetylphosphate (Sigma) at a molar ratio of 10:2:(0.5). The lipids were mixed in chloroform, then evaporated to

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dryness under nitrogen. Using a vortex mixer the residue was dispersed in aqueous isotonic media of the following compositions: (1) high potassium liposomes: KCl—130 mM, NaCl—10 mM, (2) high sodium liposomes: NaCl—140 mM, KCl—5.6 mM, (3) high sodium and EGTA liposomes: NaCl—140 mM, KCl—5.6 mM, EGTA—2 mM, (4) high calcium liposomes: $CaCl_2$—40 mM, KCl—90 mM and (5) high calcium and sodium liposomes: $CaCl_2$—40 mM, NaCl—90 mM.

In all cases the final egg PC concentration in the aqueous medium was 10 per cent (w/v). The phospholipid dispersions were sonicated to clearness (15–30 min) with a Heat System 350 W sonicator. The preparation was then centrifuged at 5000 *g* for 10 min (to remove metal particles), and then at 220,000 *g* overnight (14–16 hr). The supernatant was decanted and the precipitated vesicles were dispersed in the incubation medium (calcium-free Locke's solution with 2 mM EGTA) to a final PC concentration of 10 per cent (w/v).

RESULTS AND DISCUSSION

Incubation of adrenals in the presence of high-potassium liposomes did not affect significantly CA release (Expt. A, Fig. 1). On the other hand, incubation of adrenals in the presence of either high-calcium (Expt. B) or high sodium (Expt. C) resulted in a significant increase of CA release. The extent of increase of CA release induced by high-sodium liposomes depended on the composition of liposomes. Thus, inclusion of dicetylphosphate (DCP) in the liposomes increased substantially the effect on CA release (Expt. C) The effect of high-sodium and high-calcium liposomes on the CA release did not differ significantly (compare Expt. B with Expt. C). Moreover, there was no signficant difference between the release induced by sodium liposomes and that obtained with the sodium and calcium liposomes (Expt. E). When EGTA was added to the sodium, entrapped in high-sodium liposomes, the increased release of CA was completely blocked (Expt. D).

Incorporation of negatively-charged lipids (e.g. DCP) in liposomes has been recently reported to enhance fusion of liposomes with cells. Thus, the finding with DCP-liposomes supports the suggestion that the CA release induced by liposome–tissue interaction (in Expts. B–E) involves fusion. However, fusion of liposomes with cells, *per se*, does not trigger CA release, as is evident from the inability of high-potassium liposomes to affect CA release. Thus, the possible alteration of the cell membrane properties, due to incorporation of the liposome components into the cell membrane, is apparently inadequate for initiation of the release process. Also, the increased CA release in Expts. B–E, cannot be a result of a "leaky fusion". Such fusion would enable increased penetration of ions from the incubation medium into the adrenal cells. However, the composition of the aqueous (non-entrapped) media in

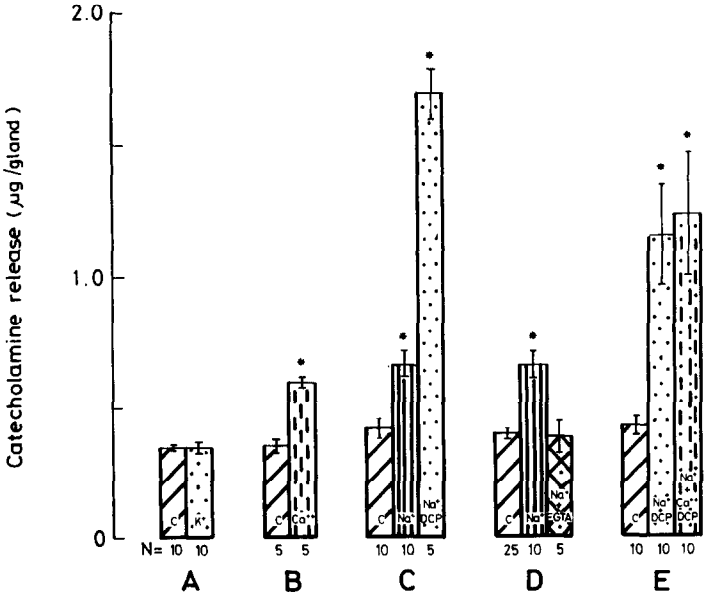

Fig. 1. Effect of liposomes loaded with various ions on catecholamine release from adrenal medulla *in vitro*.
C: release of CA without addition of liposomes.
K^+: release of CA in the presence of liposomes loaded with high-potassium medium.
Ca^{2+}: release of CA in the presence of liposomes loaded with high-calcium medium.
Na^+: release of CA in the presence of liposomes loaded with high-sodium medium.
Na^+ + DCP: liposomes loaded with Na^+ and prepared with addition of dicetylphosphate.
Na^+ + EGTA: liposomes loaded with Na^+ and EGTA (2mM).
Na^+ + Ca^{2+}: release of CA in the presence of liposomes loaded with high-sodium, high-calcium medium.
* $P < 0.01$ compared to control (C).

Adrenals were incubated for 20 min at 37° with constant shaking. A, B, C, D, E: different experiments.

all the experiments was identical, while the release of CA differed widely (Expts. A and B). Therefore, it may be assumed that the increased CA release in Expts. B–E resulted from introduction of Ca^{2+} or Na^{+} from the liposomes into the adrenal medullary cells.

The release of CA, triggered by intracellular "injection" of calcium from liposomes, may corroborate the role of calcium reported for acetycholine and other secretagogues in the adrenal medulla [21].

The effect of high-sodium liposomes cannot be ascribed to introduction of Ca^{2+}, since the liposomes were calcium-free and so was the incubation medium (EGTA was present in the medium). Increased intracellular sodium has been suggested to affect nerve and muscle cells by releasing calcium from intra-cellular organelles [3–5]. Such a mechanism is probably responsible for the induction of CA release by sodium-loaded liposomes, as indicated by the complete abolition of the effect of high-sodium liposomes by inclusion of EGTA in the sodium-liposomes (Expt. D). Furthermore, the similar effect of high sodium and calcium liposomes (Expt. E) suggests that intracellular calcium stores can provide for maximal stimulation of CA release when sodium is introduced intracellularly.

In conclusion, the present study shows that entrapment of various stimulants within liposomes can provide a tool for studying mechanisms involved in transmitter release. Increased intracellular sodium has been shown to induce CA release from adrenal medulla through mobilization of intracellular calcium stores. Experiments to elucidate the mechanism of action of other stimulants of CA release are now in progress, using the same technique.

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