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Effects of Acetylcholine and Calcium Ions on the Spontaneous Release of Epinephrine from Catecholamine Granules.* (30999)

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The recent work of Douglas and Rubin(1,2) on perfused adrenal glands has led to the hypothesis that calcium ions are involved in "stimulus-secretion coupling." They suggest that acetylcholine and/or potassium evoke adrenal medullary secretion through an action on the chromaffin cell membrane leading to an increased influx of calcium ions(3) which are the immediate stimulus for the release of the catecholamines. The stimulant effect of acetylcholine and potassium required the presence of calcium ions; the stimulant effect of calcium ions, under appropriate conditions, did not require the presence of acetylcholine or potassium.

70-80% of the catecholamine content of the adrenal medullary cell is contained within the chromaffin granules which are easily isolated from the tissue homogenates(4,5). The physiologic role of these granules is uncertain. Is their catecholamine content held primarily as a reserve store or is their catecholamine content continuously available? The morphologic basis of the catecholamine extrusion is uncertain. A number of authors have described an intracellular leakage of electron dense material, presumably the catecholamine, from the chromaffin granules(6,7,8). On the other hand, De Robertis and Vaz Ferreira(9) have described a complex chain of events during which the chromaffin granules migrate towards the cell membrane and attach themselves to it before extruding their content into the extracellular space. Thus, an agent or agents (*e.g.* acetylcholine, calcium) interacting with these granules need not penetrate the cell membrane if they are available when the granule membrane becomes continuous with cell membrane.

The effect of Ca^{++} on the release of catecholamines from bovine chromaffin granules in suspension in sucrose has been investigated with varying results. Phillipu and Schumann (10) obtained a dose related release of catecholamine from 50-150% above control with increasing concentrations of Ca^{++} from 2.5-12.5 mM. Other workers(11,12,13,14) observed a negligible release; however, they used lower concentrations of Ca^{++} in the range from 1-5 mM. Therefore, as discussed by Douglas and Rubin(2) it seemed appropriate to determine whether Ca^{++} might have an enhanced or different effect on chromaffin granules prepared in electrolyte media rather than in sucrose.

We had studied the spontaneous release of epinephrine from chromaffin granules in suspension in homogenates prepared from the adrenal gland of the rat(5). The spontaneous release was accelerated in electrolyte media and at increased temperature as had been noted with bovine(12,14) and rabbit granules (15); and acetylcholine, which depletes the adrenal gland *in vivo*, was ineffective as had been noted with bovine(10,12,16) and rabbit granules(15). However, reserpine was ineffective either as a releaser or as an inhibitor of release(5) in contrast with data obtained by others with bovine(13,17,18,19,20) and rabbit chromaffin granules(15).

In this report(21), we have examined for the effect of calcium ions, over a wide range of concentrations (.0025-25 mM), on the

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spontaneous release of catecholamines from rat adrenal medullary granules suspended in electrolyte and non-electrolyte media buffered at an acid and an alkaline pH. We have also examined whether acetylcholine in combination with calcium had a demonstrably different effect than that obtained when these are added individually.

Material and methods. The homogenates were prepared as described previously(5). Briefly, the medullae were mechanically expressed and placed in the various suspending media (0°C) in the proportion of 3 medullae/cc and gently homogenized for 45 seconds by hand in a Potter-Elvehjem homogenizer with a teflon pestle and a chamber clearance of 0.004-0.006 inches. The suspending media were: a) 125 mM KCl in 0.02 M Tris (Hydroxymethyl) Aminomethane [Tris] buffer adjusted to pH 6.5 or pH 7.4 with addition of HCl or KOH. b) 154 mM NaCl in 0.02 M Tris buffer adjusted to pH 6.5 or pH 7.4 with addition of HCl or NaOH. c) 125 mM KCl, 6 mM $MgCl_2$, 5 mM KH_2PO_4 adjusted to pH 6.5 with KOH. d) same as preceding except that the 6 mM of $MgCl_2$ were omitted. [This medium will be referred to as the Mg^{++} free phosphate buffer.] e) 300 mM sucrose medium unbuffered, pH 6.1.

Tris buffers were used because they are relatively inert with respect to calcium ions. If necessary, the pH was readjusted subsequent to the preparation of the homogenates; this was a necessary precaution since the Tris buffers are not effective at acid pH's.

0.5 cc of the various homogenates were mixed with 0.5 cc of the suspending medium (control) or the various test solutions ($CaCl_2$, acetylcholine†, Versene [EDTA]) made up in the same suspending medium and incubated for 10 minutes at 37°C. After incubation the mixtures were layered over 0.5 M sucrose and centrifuged at 20,000 *g*'s (Servall-ss-34 head) for 20 minutes at 0°C to sediment the granules. The catecholamine content of the supernatant represented the portion released, the catecholamine content of the centrifugate was presumably contributed almost entirely by the chromaffin granules, although, of course, mitochondria and other cell particulates were also present.

The data were calculated in terms of percent of epinephrine released into the supernatant of the total present in the supernatant and pellet combined. The increased or decreased release of epinephrine in each experiment was interpreted in terms of its own control value. Epinephrine and norepinephrine determinations, frequently performed in duplicate, were made by Wiegand and Perry's(22) modification of the Shore and Olin(23) fluorimetric method. Due to the large amounts of epinephrine present in relation to the norepinephrine content, we do not have confidence in the norepinephrine determinations. Therefore we are reporting only the epinephrine determinations.

Results. The adrenal medullary homogenates contained 16.8 μg/cc ($\pm$3.92) (25 determinations). Each cc contained 3 medullae estimated at 7 mg per gland. The epinephrine concentration therefore was 0.8 μg/mg net weight of the expressed tissue (which included adrenal cortical tissue as well).

The homogenates were made up in isotonic sucrose and in several different isotonic electrolyte media buffered to acid and alkaline pH's. The amount of epinephrine released spontaneously into the supernatant fluid subsequent to incubation for 10 minutes at 37°C varied from 28% in the sucrose medium (Fig. 4) to 87% in the Mg free phosphate medium (Fig 3); it was significantly decreased in the sucrose medium as compared to the others. The change in pH of the NaCl-Tris and KCl-Tris media from pH 6.5 to 7.4 had little or no influence on the percent of epinephrine spontaneously released (Fig. 1,2). The addition of 6 mM of Mg^{++} as $MgCl_2$ to the phosphate buffered medium (Fig. 3) decreased significantly the percent of epinephrine released from 87% to 75%.

The percent of the total epinephrine content spontaneously released into the supernatant fluid of the 7 control preparations incubated for 10 minutes at 37°C was: a) 76%$\pm$2.2, KCl-Tris, pH 6.5, Fig. 1; 2 determinations. b) 80%$\pm$4.2, KCl-Tris, pH 7.4, Fig. 1; 4 determinations. c) 52%$\pm$4.8, NaCl-Tris, pH

† Physostigmine (final concentration .09 mM) was added to all of the acetylcholine test solutions. Physostigmine by itself was without effect as a releaser.

6.5, Fig. 2; 3 determinations. d) 53%±5.3, NaCl-Tris, pH 7.4, Fig. 2; 6 determinations. e) 75%±1.7, phosphate medium containing 6 mM Mg^{++}, pH 6.5, Fig. 3; 4 determinations. f) 87%±2.2, Mg^{++} free phosphate medium, pH 6.5, Fig. 3; 3 determinations. g) 28%±3.8, sucrose medium, pH 6.1, Fig 4; 3 determinations.

Addition of $CaCl_2$ in final concentrations ranging from 0.0025 mM to 25 mM as noted on the abscissae (Fig. 1-4) caused slight but rather consistent effects. Each of the graphed points is the average of 2-4 separate experiments. The range of values for a given point was within the S.D. shown for the control incubations. Duplicate determinations when made were equally consistent.

Addition of $CaCl_2$ increased the percent of epinephrine released by 10-15% in the NaCl-Tris medium (Fig. 2); it decreased the percent of epinephrine released by 10-15% in the other electrolyte media both of which contained potassium as the principal cation (Fig. 1 and 3); it was ineffective in the non-electrolyte medium (Fig. 4).

The addition of acetylcholine in final concentrations ranging from 2.5-250 μg/cc was ineffective in modifying the spontaneous release of epinephrine. The percent of epinephrine released was within 1 S.D. of the control

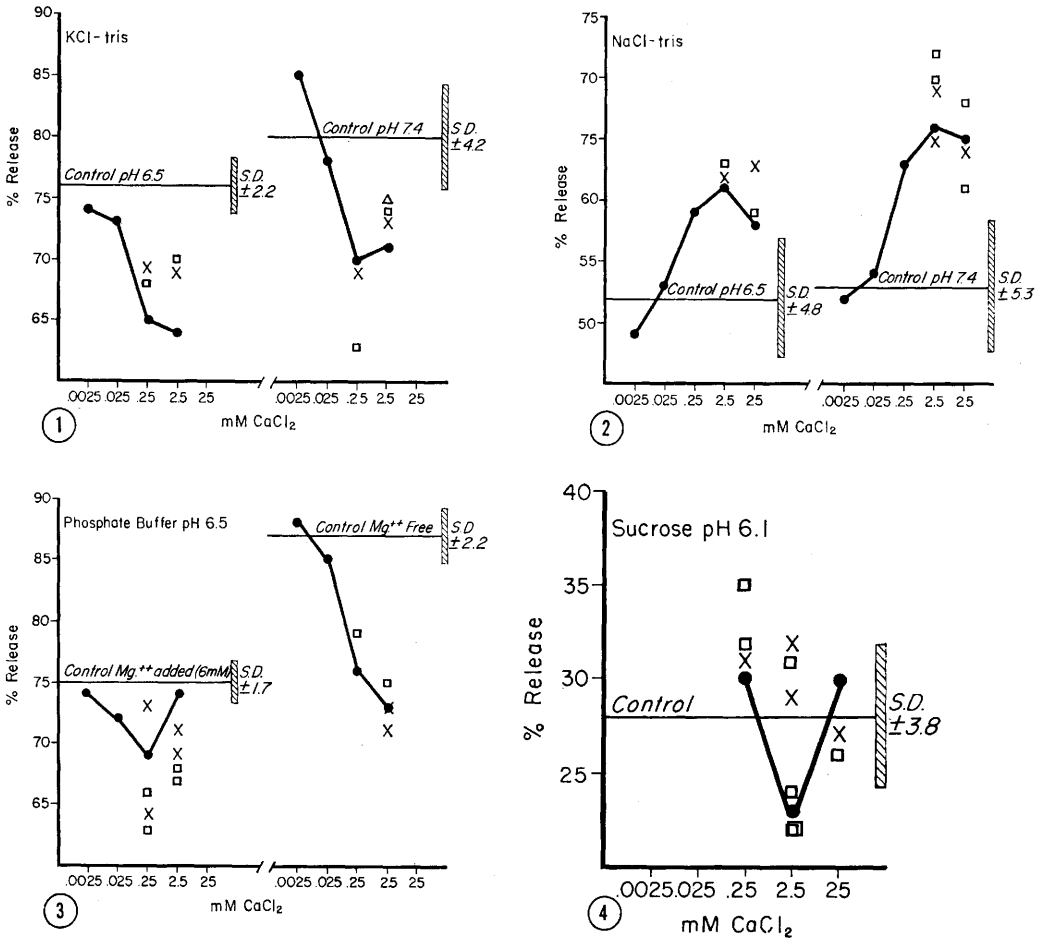

FIG. 1-4. Effect of $CaCl_2$ (filled symbols) [abscissa] on % release of epinephrine (ordinate) into the supernatant fluid from adrenal medullary homogenates made up in several different media and incubated for 10 min at 37°C. The spontaneous release (control line) and standard deviations (S.D.) in the respective media are indicated. Effects of further additions of acetylcholine (△, 2.5 μg/cc; ×, 25 μg/cc; □, 250 μg/cc) in combination with $CaCl_2$ are indicated by open symbols which represent actual concentrations within the incubation medium.

values; the data were not graphed. Addition of acetylcholine in combination with $CaCl_2$ caused slight and inconsistent modifications of the effects produced by $CaCl_2$ alone; these data are indicated by individual symbols (Fig. 1-4), each of which represents a single determination.

Addition of EDTA in final concentrations ranging from .025-.25 mM was ineffective in modifying the spontaneous release of epinephrine in the NaCl-Tris media; the data were not graphed.

Discussion. Douglas and Rubin(2) have discussed a number of possible mechanisms whereby calcium may be involved in the secretion of catecholamines. For example, based on the data of Phillipu and Schumann(10) the calcium might act directly on the chromaffin granules to cause them to release their amine content. Phillipu and Schumann(10) observed a dose related release of catecholamines from 50-150% above control with addition of 2.5-12.5 mM doses of calcium to bovine granules suspended in sucrose. Understandably, Douglas and Rubin(2) considered the above effects of calcium to be rather weak: 1) in view of the high and presumably unphysiologic concentrations of calcium that were required, 2) in view of the 1-2 times increased rate of release from the granules as compared with the many times increased secretion (100×) caused by calcium *in vivo,* 3) in view of the artificial nature of sucrose in which the granules were suspended.

Our experimental procedures were undertaken with these considerations in mind. The granule suspensions were made in a number of electrolyte media as well as in sucrose, and we investigated the effects of calcium over a greater range of concentrations. The rat adrenal gland, rather than the bovine adrenal gland, was selected because there is much more correlative experimental and histologic data available. We chose to do this study on homogenates because it enabled us to prepare the chromaffin granules in a number of different isotonic media without exposing them to the hypo-osmotic shock that occurs(24) when they are returned into isotonic media subsequent to isolation by sucrose density gradient centrifugation procedures. The use of the homogenates was further justified, despite the possibility that some of the catecholamines may have been adsorbed to other cell fragments, in that we obtained similar results with partially isolated granules in sucrose. Electronmicrographs of the pellets made from the homogenates and from the isolated preparations demonstrated the presence of many intact chromaffin granules(25).

The addition of $CaCl_2$ (.25-2.5 mM) decreased the spontaneous release of epinephrine approximately 10-15% in the media in which potassium was the major cation (Fig. 1 and 3); it increased the spontaneous release to the same extent in the media in which sodium was the major cation (Fig. 2). The opposite effects of calcium in the potassium and in the sodium containing media had not been observed previously.

Presumably, sodium and potassium are involved importantly in the mechanism of release of catecholamines in the *in vitro* system because of the marked acceleration observed as compared with the release in sucrose, and presumably calcium is involved indirectly by means of mechanisms which require the presence of sodium and potassium. Also, the effects of calcium are smaller in magnitude and they might possibly be regulatory in nature. The negligible effects of calcium in sucrose (Fig. 4) are in agreement with most previous data wherein small doses of calcium (1-5 mM) were added to suspensions of bovine granules in sucrose(11,12,13,14) with the exception of the data of Phillipu and Schumann(10). Unexplainedly, the spontaneous release from the rat granules in suspension in sucrose was unaffected at high concentrations of calcium (Fig. 4) similar to those used by Phillipu and Schumann. Presumably, the difference in the data is due to the different species used.

Addition of EDTA (.025-.25 mM) to the NaCl-Tris media should have chelated any trace quantities of calcium and should therefore have inhibited the spontaneous release of epinephrine if in fact it was being stimulated by the calcium already present. However, the added EDTA was ineffective in the NaCl-Tris medium in which addition of calcium (.25-2.5 mM) caused an increased release of catecholamines (Fig. 2).

Previously, EDTA (0.1 mM) had been reported to release epinephrine from rabbit

chromaffin granules prepared in sucrose and incubated at 37°C(26). At least two other reports (13,27) are available in which EDTA did not release epinephrine from bovine granules prepared in sucrose and incubated at 37°C; instead, the EDTA and other metal chelators caused only a slight(13) to marked inhibition(27) of epinephrine uptake, presumably by removing the magnesium or other divalent ions which are involved in epinephrine uptake.

Addition of magnesium (6 mM) (Fig. 3) to the phosphate medium diminished the spontaneous release of catecholamines from 88% to 75%. Similar findings were reported previously with rabbit chromaffin granules(15). Previously, there had been reported an increased uptake of amines with the addition of magnesium and ATP to rabbit(15) and bovine (13,27) adrenal medullary granules, and perhaps our diminished release is explainable on this basis. However, just the opposite, an increased release (2×) of catecholamines with addition of magnesium and ATP to bovine medullary granules in an electrolyte medium (14) has also been reported. We did not examine the effect of magnesium and ATP in combination.

Addition of acetylcholine (2-250 μg/ml) (final concentration) was ineffective as a releaser of catecholamines from the rat adrenal medullary granules. Similar findings had been reported previously with a variety of *in vitro* preparations (5,10,12,15,16). This correlates with the general ineffectiveness of acetylcholine as a mediator when it is injected intracellulary: *e.g.* within the muscle end-plate(28) or within the nerve axon (29,30). Acetylcholine in combination with calcium did not modify the observed effects of calcium in the electrolyte media (Fig. 1,2,3), nor did it make apparent any previously sub-minimal effect of calcium in the sucrose medium (Fig. 4). If in the organ perfusion experiments(1,2), there is a permissive interaction between acetylcholine and calcium with respect to catecholamine secretion, there was no evidence of it in the *in vitro* experiments performed by us and previously by Phillipu and Schumann (10).

The magnitude of the spontaneous release in the *in vitro* system reported by us is high as compared with the spontaneous release from the adrenal medulla *in vivo*. A similar rapid release has been demonstrated with "nerve transmitter granules" prepared from bovine splenic nerves(31). Stjarne(32) has suggested that the release from the nerve granules may be equally high *in vivo* but that it is accompanied by a very efficient reuptake mechanism involving the granule membrane as well as the plasma membrane. According to Stjarne, the rate of release and the rate of reuptake of amines by the nerve granules is 10 times higher than that observed with the adrenal medullary granules of the cow. In the cat, the adrenal gland contained high concentrations of labelled norepinephrine (several times plasma concentration) immediately after, and at 2 hours after, a quick intravenous injection(33). If we assume *in vivo* conditions of rapid release and reuptake by the adrenal gland of the rat, then the small effects of calcium (±15%) on the spontaneous release could be significant.

Summary. The amount of epinephrine released spontaneously into the supernatant from suspensions of adrenal medullary granules incubated at 37°C for 10 minutes was significantly decreased in non-electrolyte media as compared to electrolyte media. The reaction (pH 6.5-7.4) had little influence. Addition of $CaCl_2$ (.25-2.5 mM) was ineffective in the sucrose suspensions; it caused slight but consistent effects in the electrolyte media only. There was a 10-15% increased release in the sodium containing media; there was a 10-15% decreased release in the potassium containing media. Addition of acetylcholine did not modify the calcium induced effects. Addition of EDTA did not modify the spontaneous release in the sodium containing medium. The insignificant to small changes (±15%) in the spontaneous release caused by addition of calcium ions in the *in vitro* system is discussed in relation to many times (100×) increased rate of secretion caused by addition of calcium ions in organ perfusion experiments.

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A Steroidal Analgesic. (31000)

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A new conceptus concerning the structure-pharmacologic activity relationship in the central nervous system has led to the synthesis of a new class of analgesics having poly lower alkoxy estrane structures(1). These compounds, represented by *d* 2,3,4-trimethoxyestra-1,3,5(10)-triene-17β-ol (Fig. 1)*, designated MP-2001, are potent analgesics. At this time we wish to report on the pharmacology and metabolism of MP-2001.

Materials and methods. Pharmacology. All studies were carried out in propylene glycol solutions. The intravenous LD_{50} of MP-2001 was determined in Swiss-Webster mice and Wistar strain rats, utilizing the method of Bliss(2).

The drug was injected at the manually controlled rate of 1 ml/min. The oral LD_{50} was determined in mice fasted for 18 hours prior to administration of the MP-2001. Range finding studies to determine acute toxicity were also carried out in rabbits, guinea pigs, and mongrel dogs. Analgesia was studied in rats utilizing the "rat tail flick" method of D'Amour and Smith(3), as modified by Baeder. The estrogenic activity of MP-2001 was studied according to the method of Allen and Doisy(4). MP-2001 was studied for effects on blood pressure, respiration, and electro-

* M. P. 131.5-132.5°C; $[\alpha]_D^{27}$ + 74 $CHCl_3$; λ_{max}^{MeOH} 280 (E, 1857).