Mechanism of Choline-induced Secretion of Catecholamines from the Cat Adrenal Medulla: Involvement of Nicotinic Receptor

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Abstract Cat adrenal glands were retrogradely perfused *in vitro* with modified Locke's medium and the mechanism of catecholamine (CA) secretion induced by choline was investigated. Choline-induced secretion of CA was accompanied by release of dopamine- β -hydroxylase, but not by that of phenylethanolamine-*N*-methyl transferase, indicating that an exocytotic mechanism is involved in the secretion. Choline failed to induce the secretion of CA when Ca was absent from the perfusion solution. On the other hand, secretion increased when the concentration of Ca^{2+} in the perfusion medium was raised to 10 mM. Stimulation of secretion by choline was observed from a concentration of 1 mM, and half-maximal secretion occurred at about 10 mM. The stimulatory action was weaker than that of acetylcholine(ACh); the effect of 100 mM choline was approximately equal to that of 0.02 mM ACh. Secretion induced by a low concentration of choline was abolished by a nicotinic blocker, hexamethonium, while the response to choline at a concentration higher than 130 mM was only partially inhibited by cholinergic antagonists. The effects of choline and a low concentration of ACh were additive. On the other hand, 100 mM choline inhibited the response to a supra-maximal concentration of ACh(0.5 mM), suggesting that ACh and choline stimulate the same nicotinic receptor. It is concluded that choline acts partially as a nicotinic agonist and that a concentration higher than 130 mM causes secretion by a mechanism additional to nicotinic receptor activation.

Key Words: adrenal medulla, catecholamines, calcium choline, dopamine- β -hydroxylase.

We previously demonstrated that, in perfused rabbit adrenal glands, replacement of NaCl in the perfusion solution by equimolar choline chloride caused an immediate secretion of adrenaline. This secretory response could be ascribed to choline, however, the secretory effect of choline was much weaker than that

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of acetylcholine (ACh) (SORIMACHI, 1968). LASTOWECKA and TRIFARO (1974) also reported a similar finding for perfused bovine adrenal gland. Since replacement of NaCl by sucrose also induced the exocytotic secretion of catecholamines (CA), they ascribed this secretion to unknown mechanisms directly associated with Na^+ deprivation. However, they did not determine whether or not the secretory response observed in a choline-substituted medium could be inhibited by cholinergic antagonists.

We therefore carried out experiments to determine whether the secretory effect of choline can be ascribed to the interaction of choline with ACh receptors or to some other effect of Na^+ removal. The results indicate that choline is a partial nicotinic agonist, but that secretion caused by a high concentration of choline in the presence of cholinergic antagonists involves a mechanism other than nicotinic receptor activation. A preliminary account of this study has been presented at a local meeting of the Physiological Society of Japan held at Kyushu University (October 9, 1981).

MATERIALS AND METHODS

Adrenal glands of both sides of the cat were isolated and perfused at room temperature (25–30°C) in the reverse direction through the adrenolumbar vein using a procedure developed by DOUGLAS and RUBIN (1961). A flow rate of 1.0 ml/min was kept constant by means of a peristaltic pump. Samples of perfusate were usually collected in test tubes containing acetic acid. When dopamine- β -hydroxylase activity (DBH) was measured, non-acidified samples were divided into two parts; one for DBH assay was transferred to test tubes containing bovine serum albumin (final 0.25%) and the other for CA assay to test tubes containing acetic acid. Modified Locke solution of the following composition was used (mm): NaCl, 150; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; Tris/HCl (pH 7.0–7.3), 5; glucose, 5.5. When the concentration of choline chloride or CaCl_2 was increased, an osmotically equivalent amount of NaCl was omitted.

CA in the effluents was determined by use of the conventional fluorimetric method (ANTON and SAYRE, 1962). Effluents were assayed without purification on alumina, although inclusion of this step did not alter the results. Differential determination of adrenaline and noradrenaline was carried out based on the differential oxidation of the two amines at pH 7.0 and pH 3.5. DBH and phenylethanolamine-*N*-methyl transferase (PNMT) activities in the effluents were measured radiochemically using the method of MOLINOFF *et al.* (1971) as described previously (SORIMACHI and YOSHIDA, 1979) and by the method of SAAVEDRA *et al.* (1974), respectively.

RESULTS

Exocytotic nature of CA secretion caused by choline

When a control perfusion solution was switched to a solution containing

Fig. 1. Secretion of CA and DBH from the perfused adrenal gland of the cat caused by 10 mM choline or 0.05 mM ACh. The heights of the columns represent the amount of CA (μg , continuous line) and DBH (nmol/phenylethanolamine formed/hr at 37°C , dashed line) in the effluents collected every 5 min. Heavy lines under the graphs represent the periods of stimulation by choline or ACh.

10 mM choline-Cl for a short period of time, *e.g.*, 5 min, the rate of CA secretion increased immediately and returned to the prestimulation level within 10 min (Fig. 1A). When the gland was perfused with 10 mM choline for a longer period, the increased rate of secretion was not maintained at a constant level but lowered with time; the rate of secretion 30 min after the onset of stimulation declined to a level nearly the same as or a slightly higher than the initial level (Fig. 1B). This secretion pattern was quite similar to that seen in the case of ACh stimulation (Fig. 1C). The time-dependent decline of secretion during continuous stimulation by ACh has been explained by desensitization of the nicotinic receptors (KIRPEKAR and PRAT, 1978).

Secretion of CA was always accompanied by the release of DBH, but not by that of PNMT, a cytoplasmic enzyme, suggesting the exocytotic nature of the secretion. The secretion of DBH lasted for a longer period than that of CA even in the case of a shorter period of stimulation, as has often been demonstrated in the perfusion experiments (Fig. 1A; LASTOWECKA and TRIFARO, 1974; SORIMACHI and YOSHIDA, 1979; ITO *et al.*, 1980). This tendency was more pronounced with stimulation for a longer period (Fig. 1B). Since the simultaneous secretion of CA and DBH in response to ACh was observed in isolated chromaffin cells (FENWICK *et al.*, 1978), the long-lasting release of DBH seen in the present preparations may be due to the existence of vascular barriers for high molecular weight proteins.

The effect of external Ca concentration on CA secretion caused by choline

In the absence of external Ca, the secretory response to 10 mM choline was

Fig. 2. The potentiating effect of a higher concentration of Ca on CA secretion induced by 20 mM choline Cl. Left panel: a representative experiment is shown, in which an adrenal gland was perfused for 15-min periods with a medium containing Ca of 2 mM or higher before 5-min stimulation with choline (the filled rectangles under the figure). Right panel: relationship between choline-induced CA secretion and Ca concentration. The results were expressed as % of the mean of the preceding and the following responses for 2 mM Ca. Points and vertical bars represent the mean and S.E.M., respectively ($N=4$).

negligibly small ($N=3$). There was a quantitative relation between the amount of CA secreted by 10–20 mM choline and the concentration of Ca in the medium. This was shown by experiments in which responses to choline were tested alternately in a solution containing 2 mM Ca and in a solution containing a higher concentration Ca (Fig. 2, left panel). The magnitude of the response under a given concentration of Ca was expressed as a percentage of the mean of the preceding and the following ones in the presence of 2 mM Ca, and the results are summarized in the right panel of Fig. 2. CA secretion caused by choline increased as the concentration of Ca was raised. Also, CA secretion caused by ACh was reported to be dependent on the concentration of Ca (DOUGLAS and RUBIN, 1963).

The effects of various concentrations of choline on CA secretion

Choline stimulation of CA secretion was observed from a concentration of 1 mM. In order to see the dose-response relation, the responses to choline at various concentrations were tested. The responses to choline at 10 mM and at another concentration were observed alternately and the observed response was expressed as a percentage of the mean of the preceding and the following responses to 10 mM choline. As shown in Fig. 3, the response was dependent on the concentration of choline. However, the responses to 150 mM choline were much larger than those expected from the dose-response curve obtained at concentrations

Fig. 3. The effects of different concentrations of choline on CA secretion. An adrenal gland was stimulated for a 5-min period with a medium containing choline of 10 mM or another concentration alternately every 20 min, and the responses were compared to the mean of the preceding and the following ones to 10 mM choline. Points and bars represent the mean and S.E.M., respectively ($N=3-4$).

lower than 100 mM. This suggests that CA secretion caused by a high concentration of choline involves a mechanism additional to that by a low concentration of choline.

The choline-induced CA secretion in the range of choline concentration from 0 to 100 mM was significantly lower than that induced by the optimal concentration of ACh. For example, the responses to 0.01, 0.02, and 0.05 mM ACh are $55 \pm 5\%$ (mean $\pm$ S.E.M., $N=3$), $95 \pm 12\%$ ($N=3$), and $178 \pm 26\%$ ($N=3$) of the mean of the preceding and the following responses to 100 mM choline, respectively. Thus, the stimulatory effect of 100 mM choline is equivalent to that of 0.02 mM ACh. These results raise the serious question for the possibility that the secretion caused by choline is due to ACh contaminating the choline reagent. This could, however, be ruled out by the finding that alkaline treatment (pH 10.5, 60 min) of choline reagent, which destroys the ester bond of ACh, did not inhibit the response to choline (Fig. 4).

The effects of anti-cholinergic agents on CA secretion caused by choline

The secretory effect of 10 mM choline was almost completely abolished by a nicotinic blocker, hexamethonium (1.8 mM, C_6) (Fig. 5). A higher concentration of atropine (0.1 mM) also inhibited the response to choline, but a lower concentration (1.4 μ M) sufficient to block muscarinic receptors inhibited the choline-induced secretion only partially ($25 \pm 4\%$, $N=3$). Since atropine is capable of blocking nicotinic receptors as well (YOSHIZAKI, 1973; TSUJIMOTO and NISHIKAWA, 1975; TRIFARO and LEE, 1980), the role of muscarinic receptors in this secretion is obscure. Secretion caused by a low concentration of choline obviously involves C_6 -sensitive receptors, most probably nicotinic ACh receptors. These results,

Fig. 4. Effect of choline or choline plus ACh at alkaline pH on CA secretion. A solution of choline Cl dissolved in distilled water was divided into two parts: one was adjusted to pH 10.5 by adding a small amount of 1 N NaOH and the other was left at about pH 5. Each solution was further divided into two parts. Each half was given ACh and left for 60 min at room temperature. The required ions and buffer were added to these solutions before starting the experiment. Note that only the response to ACh was abolished after alkaline treatment.

Fig. 5. The effect of hexamethonium or atropine on CA secretion induced by 10 mM choline. The adrenal was treated with an agent for 15 min before 5-min stimulation.

together with the weak stimulatory effect of choline, raise two possibilities. One is that choline stimulates the secretomotor nerve terminals to induce ACh secretion. The other is that choline acts as a partial nicotinic agonist because of the lack of the acetyl group. These alternatives will be differentiated by the antagonistic effect of a high concentration of choline on ACh response (see below).

When the adrenals were stimulated with the highest concentration of choline (150 mM) in the presence of C_6 (1.8 mM) and atropine (0.1 mM), the responses were never abolished, although they were markedly reduced ($37 \pm 12\%$ of those in their absence, $N=5$). Two possibilities for this partial inhibition could be considered. Firstly, the concentration of agents employed are below the blocking doses as compared to the concentration of choline so that ACh receptors are only partially blocked. Secondly, cholinergic antagonist-resistant secretion is due to Na^+ deprivation as described earlier. In order to differentiate the two alternatives, we tested the effects of various concentrations of choline in the presence of cholinergic antagonists. These agents completely inhibited the response to a concentration of choline lower than 120 mM, but a significant increase of secretion was observed at a concentration higher than 130 mM (Fig. 6). If cholinergic antagonist-resistant secretion is related to Na^+ deficiency, replacement of 20 mM NaCl by sucrose (40 mM) in the presence of 130 mM choline should evoke more secretion. The result clearly shows that this was the case (Fig. 6, right panel). This was also confirmed by the fact that the responses to 150 mM choline were comparable to those to choline (130 mM) containing 40 mM sucrose. Consistent with this view, patterns of secretion of individual amines are different under two conditions; noradrenaline is predominantly secreted in response to 150 mM choline, but two amines are nearly equally secreted at 10 mM choline. The ratio of adrenaline to total CA is $31 \pm 4\%$ ($N=5$) in response to 150 mM choline, the values being significantly smaller than that observed at 10 mM choline ($62 \pm 3\%$, $N=14$).

Fig. 6. The effect of hexamethonium and atropine on CA secretion induced by a high concentration of choline. The left figure shows the complete inhibition of the response to 120 mM choline by cholinergic antagonists. The right figure shows that the response to 130 mM choline persists in the presence of cholinergic antagonists and this was potentiated by replacement of 20 mM NaCl with an isotonic concentration of sucrose (S).

The effect of choline on ACh-induced secretion

If the secretion caused by a low concentration of choline is due to a stimulatory effect on cholinergic nerve terminals, the effects of the submaximal concentration of ACh and choline should be additive, but this should not be the case when the concentration of ACh is supramaximal.

The responses to 10 or 100 mM choline added to 0.02 mM ACh were increased to $164 \pm 9\%$ ($N=5$) or $183 \pm 15\%$ ($N=5$) of those to ACh alone, respectively (Fig. 7, left panel). This potentiation by choline is not caused by Na^+ deficiency of the choline-containing medium, since secretion by Na^+ reduction is valid only when the concentration of Na^+ is lowered below, at least, 30 mM (Fig. 6).

On the other hand, the addition of 100 mM choline over and above the supramaximal concentration of ACh (0.5 mM) induced less CA secretion than ACh alone; the responses to ACh plus choline were reduced to $60 \pm 10\%$ ($N=4$) of those to ACh alone (Fig. 7, right panel). Choline at 10 mM did not inhibit the responses to ACh significantly ($96 \pm 8\%$ of the controls, $N=5$). Since CA secretion does not show any tendency to decline at a higher concentration of ACh (SORIMACHI, 1968; HOCHMAN and PERLMAN, 1976; HOLZ and SENTER, 1981), these results could be best explained if one considers that ACh and choline compete for the same nicotinic ACh receptors. Since the concentration of choline producing 50% of the maximal secretion is much higher (approx. 10 mM according to Fig. 4) than that to ACh (0.005–0.02 mM depending on the experiments, SCHNEIDER *et al.*, 1977; ISHIKAWA *et al.*, 1977; FENWICK *et al.*, 1978; KIDOKORO and RITCHIE, 1980; KILPATRICK *et al.*, 1980), only the higher concentration of choline would possess the capacity to replace ACh on fully saturated receptor, and thus total CA secretion would be reduced because of the weak secretory activity of choline. This view is not incompatible with the observation that choline and 0.02 mM ACh

Fig. 7. The effect of choline on the responses to the submaximal concentration (0.02 mM, left) and the supramaximal concentration (0.5 mM, right) of ACh. An adrenal gland was stimulated for a 2-min period with a medium containing ACh in the absence or presence of choline (10 or 100 mM) alternately.

are synergistic in increasing secretion, since receptors are not saturated with such a low concentration of ACh and the secretory activity of 10–100 mM choline is comparable to that of 0.01–0.02 mM ACh.

DISCUSSION

The results of the present study show that choline stimulates nicotinic receptors on the adrenomedullary cells and induces CA secretion as ACh, a physiological transmitter, does. This secretion is not caused by ACh contaminating the choline reagent. Since a high concentration of choline inhibits the maximal response to ACh, it is also unlikely that ACh either released from the secretomotor nerve terminals by choline or synthesized in these terminals from choline is responsible for this secretory response.

The results suggest that the nicotinic receptors have binding sites for choline. However, the affinity of choline for these receptors is 500–1,000 times lower than that for ACh, as was reflected by the differences in the concentrations producing 50% of the maximal secretion. As the apparent maximal response to choline is smaller than that to ACh, simultaneous bindings of the acetyl group and choline to receptors may be essential for full activation.

During the preparation of this manuscript, a paper by HOLZ and SENTER (1981) has appeared, in which they have also shown the stimulatory effect of choline on CA secretion from bovine chromaffin cells maintained in culture. Our results are in good agreement with theirs with respect to the requirement of Ca^{2+} , the minimal effective concentration of choline and the sensitivity to nicotinic blocker. However, some differences were noticed. We observed the synergistic effects of choline and submaximal concentration of ACh (0.02 mM), while they reported that, under similar experimental conditions, choline at a concentration higher than 1 mM inhibited the submaximal response to 0.03 mM ACh. The reason for this discrepancy is not clearly understood at the present time. This may be partly due to the different preparations employed. In this respect, it is of interest to note that secretion of CA from the cultured cells decreased at higher concentrations of choline (HOLZ and SENTER, 1981) and furthermore that a similar tendency was observed at higher concentrations of ACh in some experiments (SCHNEIDER *et al.*, 1977; LIANG and PERLMAN, 1979; KILPATRICK *et al.*, 1980).

In addition to its weak nicotinic activation, choline exceeding 130 mM caused secretion resistant to cholinergic antagonists. Secretion by choline substitution for Na has been interpreted as being due to Na^+ deprivation (LASTOWECKA and TRIFARO, 1974; RINK, 1977). This interpretation has to be carefully considered in two respects. Firstly, the secretion observed by them in the absence of nicotinic blocker involves that due to weak nicotinic activation. Secondly, other Na^+ substitutes such as Li^+ and Tris never induced immediate secretion, implying that secretion caused by a high concentration of choline is not simply ascribable to Na^+

removal. The mechanism of the latter secretion remains to be determined, but our results clearly indicate the necessity of the use of nicotinic blocker for this study.

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