The role of Na⁺ in muscarinic receptor-mediated catecholamine secretion in the absence of extracellular Ca²⁺ in cat perfused adrenal glands

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1 The role of Na⁺ in muscarinic receptor-mediated catecholamine secretion, which is independent of extracellular Ca²⁺, was investigated by observing the effect of veratridine and ouabain in perfused adrenal glands of the cat.

2 Veratridine (10⁻⁴ M) markedly enhanced catecholamine secretion evoked by acetylcholine (ACh, 10⁻⁴ M) during perfusion with Ca²⁺-free Locke solution containing hexamethonium (10⁻³ M). The enhancement tended to be larger for noradrenaline secretion than for adrenaline secretion. Qualitatively the same result was obtained in the response to pilocarpine (5 × 10⁻⁴ M).

3 Ouabain (10⁻⁴ M) also enhanced ACh- and pilocarpine-induced catecholamine secretions, especially noradrenaline secretion in the absence of extracellular Ca²⁺.

4 Tetrodotoxin (10⁻⁶ M) blocked the enhancing effect of veratridine on ACh-induced catecholamine secretion, but not that of ouabain in the absence of extracellular Ca²⁺.

5 When NaCl was replaced with sucrose, there was no secretory response to ACh regardless of the presence or absence of veratridine or ouabain. However, when ouabain, but not veratridine, was infused with Na⁺ before the replacement of NaCl, the response to ACh was substantially augmented.

6 These results indicate that Na⁺ is essential in the initiation of muscarinic receptor-mediated catecholamine secretion and its enhancement by veratridine and ouabain in the absence of extracellular Ca²⁺. Both drugs seem to increase the intracellular concentration of Na⁺ through different mechanisms and result in increases in the efficiency of Ca²⁺ mobilization from intracellular Ca²⁺ pools linked to muscarinic receptors.

Introduction

Catecholamine secretion induced by acetylcholine (ACh) from perfused bovine adrenal glands was inhibited, when the glands were exposed to Na⁺-free medium for a long period of time (Banks *et al.*, 1969; Lastowecka & Trifaro, 1974), whereas it was somewhat augmented after Na⁺ deprivation in cat perfused adrenal glands (Douglas & Rubin, 1963). Although the mechanisms thought to be involved in these phenomena are different and the reason for the difference between the two species remains unknown, these reports certainly indicate the importance of Na⁺ for ACh-induced release of catecholamines from adrenal chromaffin cells.

Previously, ouabain was found to enhance catecholamine secretion evoked by activation of muscarinic, but not nicotinic, receptors during perfusion with Ca²⁺-free Locke solution in cat adrenal glands (Yamada *et al.*, 1989a). We assumed that ouabain increased the intracellular concentration of Na⁺ by inhibiting the Na⁺-K⁺ pump and resulted in an increase in Ca²⁺ storage in intracellular Ca²⁺ pools linked to muscarinic receptors (Nakazato *et al.*, 1988; Yamada *et al.*, 1989a). On the other hand, the operation of Na⁺-induced Ca²⁺ release from intracellular storage sites has been suggested in many tissues (Baker & Crawford, 1975; Lowe *et al.*, 1976; Donatsch *et al.*, 1977; Silbergeld, 1977; Åkerman & Nicholls, 1981; Török, 1989). Therefore, intracellular Na⁺ may have the ability to modulate the functions of the intracellular Ca²⁺ pool. It is possible that the increase in the intracellular concentration of Na⁺ *per se* is sufficient for ouabain to enhance the catecholamine secretion evoked by muscarinic agonists, in addition to enhancement of the accumulation of Ca²⁺ in the storage sites.

Veratridine is known to increase Na⁺ permeability by decreasing the rate of inactivation of Na⁺ channels (Ohta *et*

al., 1973) and has no inhibitory action on Na⁺-K⁺ ATPase activity on nerve terminal membranes (Adam-Vizi & Ligeti, 1984). Like scorpion venom and grayanotoxin, veratridine increases catecholamine secretion from the perfused guinea-pig adrenal glands and this effect is completely blocked by either tetrodotoxin or removal of Ca²⁺, indicating the presence of Na⁺ channels on chromaffin cells similar to those on nerves (Ito *et al.*, 1978; 1979; 1980). It was reported that veratridine caused continuous secretion of catecholamines and influxes of ⁴⁵Ca²⁺ and ²²Na⁺, all of which were blocked by tetrodotoxin in bovine cultured adrenal chromaffin cells (Wada *et al.*, 1985). Thus, veratridine is a useful pharmacological tool to investigate the role of Na⁺ in adrenal catecholamine secretion.

In the present experiments, the importance of Na⁺ in catecholamine secretion induced by cholinergic agonists, especially in secretion evoked by muscarinic-receptor activation in the absence of extracellular Ca²⁺, was evaluated by studying the effect of veratridine and ouabain during perfusion with Ca²⁺-free and/or Na⁺-deficient solutions in cat adrenal glands.

Methods

Cats of either sex, weighing 1.5 to 4.5 kg, were anaesthetized with sodium pentobarbitone (40 mg kg⁻¹) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Douglas & Rubin, 1961; Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.6 to 0.8 ml min⁻¹ and maintained at room temperature (approximately 25°C). The effluent was collected continuously in 5 min aliquots into glass tubes kept on ice.

The standard perfusion medium was modified Locke solution of the following composition (mm): NaCl 154, KCl 5.6, CaCl₂ 2.2, MgCl₂ 1.2, Na₂HPO₄-NaH₂PO₄ buffer (pH 7.0) 3 and glucose 10. For Na⁺-deficient solution, NaCl was replaced by an osmotically equivalent concentration of sucrose. In the Ca²⁺-free solutions, CaCl₂ was omitted and glycoethyrdiaminetetraacetic acid (EGTA) (10⁻⁵ M) was added. Experiments were started 40 to 60 min after isolation of the adrenal gland. Secretagogues were administered for 1 min beginning 1 min before the following 5 min collection periods because of the 1 min dead time of the arterial cannula. Samples collected were acidified with 8 M perchloric acid to a final concentration of 0.4 M and stored on ice until assayed.

Adrenaline and noradrenaline were separated by high performance liquid chromatography (h.p.l.c., Jasco) and detected by an electrochemical detector (LC-4B, BAS). The detection limit was 10⁻⁸ M (0.01 nmol ml⁻¹) for either adrenaline or noradrenaline. The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982).

The following drugs were used: acetylcholine chloride (Ovisort; Daiichi), atropine sulphate (K & K), glycoethyrdiaminetetraacetic acid (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), ouabain (Tokyo Kasei), physostigmine sulphate (Wako Pure Chem.), pilocarpine hydrochloride (Tokyo Kasei), tetrodotoxin (Sankyo) and veratridine (Sigma).

Results

Effect of veratridine on the secretory responses to acetylcholine and pilocarpine in the absence of extracellular Ca²⁺

Nicotinic receptors were blocked by hexamethonium (10⁻³ M) in these experiments. A representative experiment is shown in Figure 1 and the results obtained from the same series of experiments are summarized in Table 1. First, ACh (10⁻⁴ M) was infused for 1 min during perfusion with standard Locke solution, which was then changed to Ca²⁺-free Locke solution containing EGTA (10⁻⁵ M). In the presence of Ca²⁺, ACh caused an increase in both adrenaline and noradrenaline secretions. The ratio of adrenaline to noradrenaline released was about 1:1 (Table 1). Fifteen minutes after the removal of Ca²⁺, the adrenal glands were again stimulated with ACh. Under these conditions, ACh still caused a small increase in catecholamine secretion. As reported previously (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1989a), this residual response was repeatedly obtained as long as the adrenal gland was loaded with Ca²⁺ prior to stimulation with ACh. Veratridine (10⁻⁴ M) was added to the perfusion solution 5 min after the end of Ca²⁺ loading for 25 min and the gland was stimulated with ACh (10⁻⁴ M) for 1 min, 10 min after the start of veratridine infusion. Veratridine markedly enhanced ACh-induced catecholamine secretion and the magnitude of the restored

Figure 1 Effect of veratridine on acetylcholine-evoked catecholamine secretion in the absence of extracellular Ca²⁺. Acetylcholine (ACh, 10⁻⁴ M) was sequentially applied for 1 min during perfusion with standard Locke solution or Ca²⁺-free Locke solution containing 10⁻⁵ M EGTA. Veratridine (10⁻⁴ M) was infused during the period indicated by the horizontal bar delimited by arrows above the histograms. Hexamethonium (10⁻³ M) was present throughout the experiment. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

Table 1 Catecholamine secretions induced by acetylcholine (ACh) in the presence (A) and absence (B) of extracellular Ca²⁺ before and during treatment with veratridine (10⁻⁴ M) in cat perfused adrenal glands

	(A) Locke		(B) Ca ²⁺ -free Locke			
	Ad	NA	Control	Veratridine 10 ⁻⁴ M	Control	Veratridine 10 ⁻⁴ M
	Ad	NA	Ad	NA	Ad	NA
Resting release	6.3 ± 2.7	4.2 ± 1.4	0.5 ± 0.1	0.5 ± 0.1	1.3 ± 0.4	2.2 ± 0.4
ACh 10 ⁻⁴ M	13.3 ± 2.0	12.5 ± 2.2	1.3 ± 0.2	2.2 ± 0.4	12.0 ± 3.4	14.1 ± 1.6
No. of cats	7	7	9	9	3	3

Values indicate the mean (± s.e.) of adrenaline (Ad) and noradrenaline (NA) released in nmol 5 min⁻¹ and the percentage of noradrenaline is given in parentheses under the various conditions indicated. The values of ACh-induced catecholamine secretions were obtained by subtracting the values of resting release from the respective evoked release of adrenaline and noradrenaline. In Ca²⁺-free Locke solution, CaCl₂ was omitted and 10⁻⁵ M EGTA was added. All solutions contained hexamethonium (10⁻³ M).

responses attained almost the same level as those obtained in the presence of extracellular Ca²⁺ (Table 1). The percentage of noradrenaline released was increased a little. ACh applied 15 min after the first challenge failed to induce a response, even if veratridine was still present. As shown in Table 1, veratridine also caused a small increase in resting catecholamine secretion in the absence of extracellular Ca²⁺. When 10⁻⁵ M veratridine was used, the response to ACh was also enhanced, but to a lesser extent.

In a similar fashion, the effect of veratridine on catecholamine secretion induced by pilocarpine was investigated without adding hexamethonium. In a manner similar to ACh-induced catecholamine secretion, veratridine also enhanced the secretory response to pilocarpine, especially noradrenaline secretion (Figure 2).

Effect of ouabain on the secretory responses to acetylcholine and pilocarpine in the absence of extracellular Ca²⁺

The effect of ouabain (10⁻⁴ M) on catecholamine secretion evoked by muscarinic receptor activation was studied with a protocol similar to that for veratridine. As indicated in Figure 3a, catecholamine secretion induced by ACh (10⁻⁴ M) was enhanced by ouabain infused in the absence of extracellular Ca²⁺. Unlike results with veratridine, the enhancement was greater in the response to the second challenge with ACh than to the first one. The response to pilocarpine (5 × 10⁻⁴ M) was also enhanced by ouabain (Figure 3b). The enhancement of noradrenaline secretion was much larger than that of adrenaline secretion in response to either ACh or pilocarpine.

The enhancement of ACh- and pilocarpine-induced secretory responses by veratridine and ouabain in the absence of extracellular Ca²⁺ was completely blocked by atropine (10⁻⁶ M) regardless of the presence or absence of hexamethonium (10⁻³ M).

Effects of tetrodotoxin on the enhancement of ACh-induced catecholamine secretion by veratridine

To confirm the involvement of voltage-dependent Na⁺ channels in the enhancement by veratridine, the effect of tet-

Figure 2 Effect of veratridine on pilocarpine-evoked catecholamine secretion in the absence of extracellular Ca²⁺. Pilocarpine (Pilo, 5 × 10⁻⁴ M) was sequentially applied for 1 min during perfusion with standard Locke solution or Ca²⁺-free Locke solution containing 10⁻³ M EGTA. Veratridine (10⁻⁴ M) was infused during the period indicated by the horizontal bar delimited by arrows above the histograms. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

Figure 3 Effect of ouabain on catecholamine secretions evoked by acetylcholine and pilocarpine. Acetylcholine (ACh, 10⁻⁴ M) (a) and pilocarpine (Pilo, 5 × 10⁻⁴ M) (b) were sequentially applied for 1 min during perfusion with standard Locke solution and/or Ca²⁺-free Locke solution containing 10⁻³ M EGTA. Ouabain (10⁻⁵ M) was infused during the period indicated by the horizontal bar delimited by arrows above the histograms. Hexamethonium (10⁻³ M) was present throughout the experiment in (a). Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

rodotoxin on catecholamine secretion evoked by muscarinic receptor activation was studied. A representative result is illustrated in Figure 4. First, control responses to ACh (10⁻⁴ M) were successively obtained in the presence and absence of extracellular Ca²⁺, then the adrenal gland was loaded with Ca²⁺ for 25 min. Fifteen minutes after the end of Ca²⁺ loading, ACh was again administered in the presence of veratridine (10⁻⁴ M) plus tetrodotoxin (10⁻⁶ M). Tetrodotoxin completely inhibited the enhancing effect of veratridine on ACh-induced catecholamine secretion that was unmasked after the removal of the toxin (Figure 4). On the other hand, tetrodotoxin failed to inhibit the enhancement of ACh-induced catecholamine secretion by ouabain (10⁻⁴ M) (data not shown).

Effect of Na⁺ deprivation on muscarinic receptor-mediated catecholamine secretion

Experiments were carried out to determine whether the catecholamine secretion evoked by muscarinic receptor activation in the absence of extracellular Ca²⁺ could be observed in the absence of Na⁺. Since Na⁺ deprivation causes a marked increase in catecholamine secretion (Lastowecka & Trifaro,

Figure 4 Effect of tetrodotoxin on the enhancement of acetylcholine-induced catecholamine secretion by veratridine. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min during perfusion with standard Locke solution or Ca^{2+} -free Locke solution containing 10^{-5} M EGTA. Veratridine (10^{-4} M) and tetrodotoxin (TTX, 10^{-6} M) were infused during the periods indicated by the horizontal bars delimited by arrows above the histograms. Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

1974; Ito *et al.*, 1979), the glands were challenged with ACh (10^{-4} M) after the termination of the Na^{+} -deficient response. As shown in Figure 5, ACh failed to increase catecholamine secretion during perfusion with Na^{+} -deficient solution, but the response to ACh was completely restored to the control value after readmission of Na^{+} .

Requirement of Na^{+} for the enhancement of muscarinic receptor-mediated catecholamine secretion by veratridine and ouabain

To study the requirement of Na^{+} for the enhancement of ACh-induced catecholamine secretion by veratridine and ouabain, ACh (10^{-4} M) was applied during perfusion with Ca^{2+} -free, Na^{+} -deficient solution containing veratridine (10^{-4} M) or ouabain (10^{-4} M). Under these conditions, both drugs failed to enhance ACh-induced catecholamine secretion (data not shown). Then veratridine or ouabain was infused for 25 min with both Ca^{2+} and Na^{+} , and ACh (10^{-4} M) was applied 25 min after removal of the drugs and the cations. A representative experiment is illustrated in Figure 6, where veratridine (10^{-4} M) and ouabain (10^{-4} M) were sequentially infused at intervals of 95 min into the same adrenal prep-

aration. Veratridine and ouabain caused a remarkable increase in catecholamine secretion (not illustrated in the figure) which quickly declined after the removal of the drugs and cations until it attained a new steady level. As shown in Figure 6, the response to ACh in the Ca^{2+} -free, Na^{+} -deficient environment was only slightly enhanced after treatment with veratridine, but was markedly increased after ouabain treatment. Finally, either veratridine or ouabain was infused for 25 min in the presence of Na^{+} immediately after the end of Ca^{2+} loading, and ACh was administered 25 min after the removal of the drug and NaCl. As in the preceding experiments, ACh-induced catecholamine secretion was enhanced by ouabain, but not by veratridine. The effect of ouabain is illustrated in Figure 7.

Discussion

The present experiments show that Na^{+} is essential for ACh to release catecholamine from cat adrenal glands through activation of muscarinic receptors in the absence of extracellular Ca^{2+} . Na^{+} seems to play an important role in increasing the efficiency of ACh-induced Ca^{2+} mobilization from intracellular Ca^{2+} pools, which are postulated to link to muscarinic

Figure 5 Catecholamine secretion evoked by acetylcholine during perfusion with Ca^{2+} -free, Na^{+} -deficient solution. Acetylcholine (ACh, 10^{-4} M) was sequentially applied for 1 min in the presence or absence of Ca^{2+} and/or Na^{+} . Hexamethonium (10^{-3} M) was present throughout the experiment. In Ca^{2+} -free solutions, CaCl_2 was omitted and 10^{-5} M EGTA was added. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

Figure 6 Catecholamine secretions evoked by acetylcholine during perfusion with Ca^{2+} -free, Na^+ -deficient solution after exposure to veratridine and ouabain with Ca^{2+} and Na^+ . Acetylcholine (ACh , 10^{-4} M) was sequentially applied for 1 min in the presence and absence of Ca^{2+} and/or Na^+ . In Ca^{2+} -free solutions, CaCl_2 was omitted and 10^{-5} M EGTA was added. Veratridine (10^{-4} M) and ouabain (10^{-4} M) were sequentially infused for 25 min at intervals of 95 min in the presence of both Ca^{2+} and Na^+ . Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the amounts of adrenaline (open) and noradrenaline (hatched) released in nmol per 5 min.

receptors. The effects of veratridine, ouabain and Na^+ deprivation provided some evidence supporting this view. First, catecholamine secretion induced by ACh was reversibly inhibited by Na^+ deprivation during perfusion with Ca^{2+} -free Locke solution. Second, veratridine and ouabain enhanced catecholamine secretions evoked by ACh and pilocarpine during perfusion with Ca^{2+} -free Locke solution. These potentiating effects were inhibited by removal of Na^+ . Third, pretreatment with ouabain plus Na^+ with or without Ca^{2+} enhanced the secretory response to ACh obtained subsequently during perfusion with Ca^{2+} -free, Na^+ -deficient solution.

It has been suggested that the increase in the intracellular concentration of Na^+ releases Ca^{2+} from internal stores in several systems (Baker & Crawford, 1975; Lowe *et al.*, 1976; Donatsch *et al.*, 1977; Silbergeld, 1977; Åkerman & Nicholls, 1981). The Na^+ -induced Ca^{2+} release mechanism was also

reported to operate in adrenal chromaffin cells (Gutman *et al.*, 1979). In the present study, spontaneous catecholamine secretion was only slightly enhanced by veratridine in the absence of extracellular Ca^{2+} , in contrast to the marked enhancement of muscarinic receptor-mediated secretory response. Therefore, the Na^+ -induced Ca^{2+} release mechanism does not seem to play a major role in releasing spontaneous catecholamine secretion from adrenal chromaffin cells, if it does exist.

Previously, we found that ouabain enhanced catecholamine secretions evoked by splanchnic nerve stimulation, ACh and high K^+ in perfused guinea-pig adrenal glands (Nakazato *et al.*, 1986). In this previous work, we already noticed that the high K^+ -induced secretory response enhanced by ouabain was completely inhibited by removal of extracellular Ca^{2+} and addition of Mg^{2+} , Co^{2+} or Ni^{2+} , but the secretory response to ACh was relatively resistant to these treatments. Later, it was found that the activation of muscarinic, but not

Figure 7 Catecholamine secretion evoked by acetylcholine during perfusion with Ca^{2+} -free, Na^+ -deficient solution after exposure to ouabain with Na^+ . Acetylcholine (ACh , 10^{-4} M) was sequentially applied for 1 min in the presence or absence of Ca^{2+} and/or Na^+ before and after exposure to ouabain (10^{-4} M) in Ca^{2+} -free Locke solution. In Ca^{2+} -free solutions, CaCl_2 was omitted and 10^{-5} M EGTA was added. Hexamethonium (10^{-3} M) was present throughout the experiment. Columns show the means of adrenaline (open) and noradrenaline (hatched) released in 5 experiments; vertical bars show s.e.mean.

nicotinic, receptors was partially effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} (Nakazato *et al.*, 1984; 1988). Furthermore, we reported that ouabain enhanced the secretory responses to stimulation of nicotinic and muscarinic receptors in the presence of extracellular Ca^{2+} , though only those induced by muscarinic receptor activation in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1989a). Our interpretation is that ouabain increases the intracellular concentration of Na^+ by inhibiting the Na^+ - K^+ pump and results in an increase in the entry of Ca^{2+} in response to nicotinic stimulation or high K^+ , and that at the same time ouabain causes an increase in storage of Ca^{2+} in intracellular Ca^{2+} pools linked to muscarinic receptors. Veratridine differs from ouabain in that it increases the entry of Na^+ through the activation of tetrodotoxin-sensitive Na^+ channels. Despite this fact, the effect of veratridine obtained in the present study suggests that the increase in the intracellular concentration of Na^+ augments the efficiency of the release of Ca^{2+} from internal stores and causes the potentiation of muscarinic receptor-mediated catecholamine secretion. This Na^+ -dependent mechanism seems to be more marked in noradrenaline cells than in adrenaline cells, because both veratridine and ouabain increased noradrenaline secretion more than adrenaline secretion in response to ACh in the absence of extracellular Ca^{2+} .

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- Although a role for inositol 1,4,5-triphosphate has been suggested in bovine adrenal chromaffin cells (Stoehr *et al.*, 1986), there is little available information on the properties of intracellular Ca^{2+} pools responsible for agonist-induced catecholamine secretion. In previous works, we found that caffeine, which is known to enhance Ca^{2+} -induced Ca^{2+} release from sarcoplasmic reticulum of muscles (Endo, 1977), was effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} in perfused adrenal glands of the cat (Yamada *et al.*, 1988; 1989b). The catecholamine secretion induced by both caffeine and ACh was reversibly blocked by the intracellular Ca^{2+} antagonist TMB-8 (Yamada *et al.*, 1988) and was enhanced by ouabain (Yamada *et al.*, 1989a,b). However, pretreatment with caffeine did not entirely inhibit the subsequent secretory response to ACh (Yamada *et al.*, 1989b). Therefore, as in smooth muscle where Ca^{2+} stores consist of two classes (Iino *et al.*, 1988), adrenal chromaffin cells may possess heterogeneous Ca^{2+} pools in which the capacity to store Ca^{2+} and/or the ability to release Ca^{2+} is under the control of intracellular Na^+ .
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