EFFECTS OF NICOTINIC RECEPTOR AGONISTS AND
ANTAGONISTS ON UPTAKE AND EFFLUX OF 45Ca
IN PERFUSED BOVINE ADRENAL GLANDS

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Abstract—The actions of nicotinic receptor agonists and antagonists on Ca movement in perfused bovine adrenal glands were investigated. 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM) and acetylcholine (ACh, 0.3 mM, containing 10 nM physostigmine) produced a significant increase in 45Ca efflux from 45Ca preloaded adrenal glands and which was closely correlated with an increase of adrenal catecholamine (CA) secretion. In the perfused adrenal glands which the stimulation of 45Ca efflux as well as adrenal CA secretion by ACh was abolished in Ca free Locke’s solution, the application of Ca (22 mM) considerably restored the stimulatory response of 45Ca efflux to ACh. There was a significant increase in the 45Ca uptake by nicotinic agonists-stimulated adrenal glands, which was responsible for the enhancement of Ca uptake by adrenal medulla and not by the cortex. The maximal increase in 45Ca uptake by nicotinic agonists preceded that in the adrenal CA secretion. Nicotinic antagonists such as surugatoxin (SGTX, 20 nM) and mecamylamine (0.1 mM) markedly (60-90%) reduced the stimulation of uptake and efflux of 45Ca by the agonists. Thus the present study has demonstrated that nicotinic agonists-induced CA secretion may be associated with a stimulation of uptake and efflux of Ca in bovine adrenal medulla and that nicotinic antagonists may inhibit the stimulated flux of Ca by the action on nicotinic receptors of adrenal chromaffin cells.

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The importance of calcium ions (Ca) in the release of neurotransmitters and hormones has been well established (1). Secretion of catecholamines (CA) from adrenal glands elicited by acetylcholine (ACh) or high potassium requires the presence of Ca in the extracellular medium (2–4). Since an increased influx of radioactive Ca into the cat adrenal medulla occurs during the stimulation with ACh (5–7), the entry of Ca into the medullary chromaffin cells has been shown to initiate the secretory process (5, 8–10). Using bovine adrenal glands which were perfused with the Locke’s solution containing 45Ca and ACh, Borowitz (11) has demonstrated that ACh significantly increased the 45Ca remaining in medullary tissue after the washout of extracellular 45Ca. However, there was no significant alteration in the total Ca content in ACh-stimulated glands which was determined by atomic absorption spectroscopy (11). Since both tetracaine and Mg++ which inhibited adrenal CA secretion markedly depressed Ca exchange between the perfused cat adrenal
gland and the perfusate (7), adrenal CA secretion may be associated with a significant stimulation of Ca movement. Thus, we examined the action of nicotinic receptor agonists and antagonists on the uptake and efflux of Ca using ⁴⁵Ca with the simultaneous determination of CA secretion in perfused bovine adrenal glands.

MATERIALS AND METHODS

Perfusion of bovine adrenal glands in vitro: Bovine adrenal glands (weighing 9-15 g) obtained at the local slaughter house were kept in ice with a few minutes of their removal from animals and prepared for the perfusion. A polyethylene cannula was placed in the adrenal central vein and the glands were perfused in retrograde fashion (8) at room temperature (22-25°C) with Locke's solution, using a multichannel metering pump (Harvard Apparatus Co., Inc., Millis., Mass.). The flow rate during the perfusion was constantly maintained at 4 ml/min. The glands were perfused for 40 min in order to allow CA secretion to reach steady values and to wash out the blood. The venous samples were collected at 2- or 3-min intervals and CA were estimated according to the fluorometric method described by Anton and Sayre (12), using adrenaline and noradrenaline as standards. The composition of the Locke's solution was as follows (mM): NaCl, 154; KCl, 5.6; CaCl₂, 2.2; Na₂HPO₄, 2.2; Na₂HPO₄, 0.85 and glucose, 10. In calcium free Locke's solution, CaCl₂ was omitted and EGTA 1 mM was present. High potassium Locke's solution contained 56 mM KCl with the decreased concentration of NaCl to maintain isotonicity. All these solutions were equilibrated with 5% CO₂ in O₂ and maintained at pH 7.2. The agonists were mostly infused during a period of 3 min into the perfusion fluid at the point of a cannula inserted into the adrenal glands. Cumulative concentration-effect curves were constructed for DMPP with three doses. In some experiments, CA secretion was evoked by switching perfusion from Locke's solution to the solution containing the agonists for periods of 15 min. The nicotinic antagonists were added to the perfusion fluid 15 min before and during the stimulation. The agonist-induced CA secretion was corrected for the spontaneous secretion (29.3±1.4 μg/3 min, n=91).

Experiments with ⁴⁶Ca flux: In the experiments with ⁴⁵Ca efflux, adrenal glands were perfused with Ca free Locke's solution for 25 min, followed by a 15-min infusion of ⁴⁶Ca (2.2 mM, 1 μCi/ml; specific activity, 11.3 mCi/mg) and an addition of 30-min washout with the ordinary solution to remove the extracellular ⁴⁵Ca. The glands were then stimulated with agonists for 15 min and the radioactivity as well as CA concentration in the effluents was determined. The data for ⁴⁶Ca efflux were analyzed by plotting the radioactivity in the effluents against the time following the perfusion with non-radioactive Ca Locke's solution.

In the experiments with ⁴⁵Ca uptake, Ca uptake by adrenal glands was determined from the decrease of radioactivity in the effluents during the perfusion with Locke's solution containing ⁴⁵Ca. Bovine adrenal glands were perfused by Locke's solution containing ⁴⁵Ca (0.015 μCi/ml) in the absence and presence of agonists for 15 min and the radioactivity in the effluents was determined every 1 or 2 min. Thus the amount of Ca uptake by perfused adrenal glands was determined from the decreased amount of ⁴⁵Ca in the effluents during the perfusion with ⁴⁵Ca as compared to the preperfusates. The amount of ⁴⁵Ca uptake by agonist stimulation was determined from the difference in the radioactivity in the effluents between the absence (spontaneous) and presence of the agonist. The effluents...
were collected in liquid scintillation vials and after the addition of 5 ml scintillation cocktail, the radioactivity was measured with an liquid scintillation counter (Aloka). To determine $^{45}$Ca uptake by the adrenal medulla and cortex, adrenal glands were perfused with Locke's solution containing $^{45}$Ca (0.1 μCi/ml) for 15 min, and during the perfusion, a 3-min infusion of agonists were given intermittently at 2 min intervals. Control adrenal glands were not stimulated by agonists. The adrenal glands were then washed by perfusion with Ca free Locke's solution (containing 1 mM EGTA). After the 48 min washout, adrenal glands were separated into the medulla and cortices, and the $^{45}$Ca remaining in both tissues after wet-digesting with HNO$_3$ and H$_2$O$_2$ was measured. The counting cocktail consisted of 1% 2,5-diphenyloxazole (PPO), 0.025% 1,4-bis (2-(5-phenyloxazolyl)) benzene (POPOP) and 10% naphthalene in dioxane. In the experiments with $^{45}$Ca flux, a simultaneous measurement of CA in the effluents was performed.

The drugs used were obtained from the following sources: acetylcholine chloride (Daichi), histamine diphosphate (Wako), nicotine tartrate (Wako), 1,1-dimethyl-4-phenylpiperazinium iodide (Aldrich), hexamethonium chloride (Wako), mecamylamine hydrochloride (Sigma), physostigmine sulphate (Merck), EGTA (Kanto). $^{45}$Ca was obtained from New England Nuclear, Boston, Mass. and SGTX was provided by Prof. T. Kosuge of this college. Statistical analysis of data was performed using a double-tailed Student's t-test.

RESULTS

Effects on catecholamine secretion: The effects of nicotinic receptor agonists and antagonists on CA secretion from perfused bovine adrenal glands were examined. 1,1-dimethyl-4-phenylpiperazinium (DMPP) (40 μM-1 mM), ACh (40 μM-1 mM, containing 10 μM physostigmine) and nicotine (0.2–1 mM) stimulated the CA secretion from perfused bovine adrenal glands, which was abolished by the perfusion of adrenal glands with Ca free Locke's solution containing 1 mM EGTA. The stimulatory effects by these agonists were the concentration-dependent as shown in Fig. 1 (for DMPP). The mean increase in CA secretion by DMPP, ACh and nicotine at the concentration of 0.2 mM was 93.7±17.1, 80.3±12.4 and 68.1±10.1 μg/3 min (n=6–16), respectively. The adrenal CA secretion by these agonists was significantly reduced by the pretreatment with nicotinic antagonists. Surugatoxin (SGTX, 20 μM) which has been demonstrated to be a specific antagonist of nicotinic receptors in superior cervical ganglia of cats (13) and rats (14), significantly reduced DMPP-
ACh- and nicotine-induced CA secretion from perfused bovine adrenal glands (Fig. 1). The inhibition rate (mean±S.E., N=3-5) to 1 mM of DMPP, ACh and nicotine was 70.2±5.4%, 63.8±3.1% and 71.0±5.2%, respectively. On the other hand, SGTX in this concentration had little effect on the spontaneous CA secretion and on the histamine (0.4 mM)- and high potassium (56 mM)-induced CA secretion. Similar inhibition (60-90%, N=3 or 4) of DMPP- and ACh-induced CA secretion by hexamethonium (1 mM) and mecamylamine (0.1 mM) was observed.

Effects on $^{45}$Ca efflux: The $^{45}$Ca efflux from $^{45}$Ca preloaded adrenal glands showed an exponential decline with initial rapid phase which was followed by a slower phase after about 26 min. The slower phase may reflect the $^{45}$Ca efflux from cellular sites as previously reported (6, 7). Thus the effects of nicotinic agonists and antagonists on the $^{45}$Ca efflux

![Fig. 2](image)

**Fig. 2.** Effect of ACh and mecamylamine on $^{45}$Ca efflux from perfused bovine adrenal glands. a), A typical stimulatory effect of ACh (0.3 mM, containing 10 μM physostigmine: Ese) on $^{45}$Ca efflux. b), A typical inhibitory effect of mecamylamine (0.1 mM) on ACh-stimulated $^{45}$Ca efflux. $^{45}$Ca efflux was expressed as dpm/ml of the perfusate. Column represents ACh-induced catecholamine secretion (μg/2 min).

| Table 1. Effect of nicotinic agonists and antagonists on $^{45}$Ca efflux and catecholamine secretion from perfused bovine adrenal glands preloaded with $^{45}$Ca. |
|-----------------|-----------------|-----------------|
|                  | $^{45}$Ca efflux ($\times 10^6$ dpm/15 min) | Catecholamine secretion (μg/15 min) |
| DMPP (0.3 mM)   | 7.14±1.90       | 285±22          |
| DMPP (0.3 mM)+SGTX (20 μM) | 1.52±0.48*     | 59.3±10.1**     |
| DMPP (0.3 mM)+mecamylamine (0.1 mM) | 1.62±0.47*     | 68.0±9.9**      |
| ACh (0.3 mM)    | 9.78±2.58       | 256±24          |
| ACh (0.3 mM)*+mecamylamine (0.1 mM) | 0.19±0.04*     | 66.0±15.1**     |

Each value represents the mean (±S.E., 3 adrenal glands) of total $^{45}$Ca efflux ($\times 10^6$ dpm/15 min) or catecholamine secretion (μg/15 min) during the 15 min perfusion of bovine adrenal glands with the Locke's solution containing nicotinic agonists or antagonists. Asterisks show a significant difference from each control value, *P<0.05, **P<0.01. * containing 10 μM physostigmine.
were examined 30 min after the washout. The perfusion with DMPP (0.3 mM) and ACh (0.3 mM, containing 10 μM physostigmine) markedly increased the 45Ca efflux from 45Ca preloaded adrenal glands mostly up to 2 or 3 times the prestimulation level within 8–10 min and thereafter declined. Figure 2 and table 1 illustrate a typical example for the stimulation of 45Ca efflux by ACh (0.3 mM, containing 10 μM physostigmine) and the increase in 45Ca efflux and CA secretion during the 15 min perfusion with DMPP or ACh, respectively. The stimulation of 45Ca efflux by these nicotinic agonists was accompanied by a marked increase in CA secretion from adrenal glands (Fig. 2a, for ACh) and completely inhibited by the perfusion with Ca free Locke’s solution (Fig. 3). During the perfusion of adrenal glands with Ca free Locke’s solution containing ACh (0.3 mM), the application of high Ca (22 mM, 4 min) considerably restored the stimulation of 45Ca efflux and CA secretion by ACh as shown in Fig. 3 (increase in 45Ca efflux and CA secretion: 1.77±0.05 μmol/4 min, 224±29 μg/4 min, N=4). However the application of this concentration of Ca in the normal Locke’s solution (containing 2.2 mM Ca) did not affect the 45Ca efflux and CA secretion (data not shown). As shown in Table 1, the stimulatory effects of 45Ca efflux as well as CA secretion by DMPP (0.3 mM) and ACh (0.3 mM) were markedly inhibited by the preperfusion with SGTX (20 μM) or mecamylamine (0.1 mM). A typical example for the inhibition of ACh stimulation by mecamylamine (0.1 mM) is illustrated in Fig. 2b.

Effects on 45Ca uptake: The amount of 45Ca uptake by agonist-stimulated adrenal glands was determined from the difference in the radioactivity in the effluents between absence (spontaneous) and presence of agonists during the perfusion of bovine adrenal glands with 45Ca. Figure 4 and Table 2

![Fig. 3. Effect of Ca omission on the stimulation of 45Ca efflux by ACh from the perfused bovine adrenal gland preloaded with 45Ca, and the stimulation by Ca. Adrenal gland preloaded with 45Ca was washed out with Ca free Locke’s solution for 30 min and then stimulated with ACh (0.3 mM) in the presence of physostigmine (10 μM Ese). A 4-min infusion of Ca (22 mM) was performed 14 min after the ACh perfusion. 45Ca efflux was expressed as dpm/ml of the perfusate. Column represents Ca-induced catecholamine secretion (μg/2 min).]
show the increased uptake of net Ca by the ACh (0.3 mM)- and DMPP (0.3 mM)-stimulated adrenal gland which was determined from the amount of influxed $^{45}$Ca. Thus the 15 min perfusion of bovine adrenal glands with ACh (0.3 mM, containing 10 μM

Fig. 4. Effect of ACh and mecamylamine on Ca uptake by perfused bovine adrenal glands. The ordinate represents ACh-stimulated uptake of net Ca which was determined from the amount of influxed $^{45}$Ca and the abscissa shows the time (min) after initiating ACh stimulation. •—•, ACh (0.3 mM, containing 10 μM physostigmine: Ese); △—△, mecamylamine (0.1 mM)+ACh (0.3 mM, containing 10 μM physostigmine). Ca uptake was expressed as pmol/mg wet weight/sec or μmol/min. Column represents the ACh-induced catecholamine secretion (μg/2 min) (open column, ACh; hatched column, mecamylamine+ACh). Each point represents the mean±S.E. from 4 adrenal glands.

Table 2. Effect of nicotinic agonists and antagonists on Ca uptake and catecholamine secretion by bovine adrenal glands

|                        | Total Ca uptake (μmol/15 min) | Catecholamine secretion (μg/15 min) |
|------------------------|-------------------------------|------------------------------------|
| DMPP (0.3 mM)          | 13.8±2.4                      | 328±19                             |
| DMPP (0.3 mM)+SGTX (20 μM) | 5.23±0.92*                 | 63.7±16.7**                        |
| DMPP (0.3 mM)+mecamylamine (0.1 mM) | 6.37±1.30*             | 84.7±13.0**                        |
| ACh (0.3 mM)            | 10.3±1.9                      | 295±17                             |
| ACh (0.3 mM)+mecamylamine (0.1 mM) | 3.44±0.89*                 | 66.0±15.2**                        |

$^{45}$Ca uptake by nicotinic agonists during the perfusion of bovine adrenal glands with Locke's solution was measured as described in Methods. Each value represents the mean (±S.E., 3 or 4 adrenal glands) of total Ca uptake (μmol/15 min) or catecholamine secretion (μg/15 min) by bovine adrenal glands perfused for 15 min with Locke's solution containing nicotinic agonists and nicotinic antagonists. Asterisks show a significant difference from each control value. *P<0.05, **P<0.01. a containing 10 μM physostigmine.
physostigmine) significantly increased the Ca uptake by adrenal glands. Since the increase in Ca uptake was maximal around 2 min following the perfusion with ACh (0.3 mM) and thereafter declined to become a constant level, the stimulated uptake of Ca by ACh appears to precede the maximal increase in adrenal CA secretion (Fig. 4). Similarly, the perfusion with DMPP (0.3 mM) stimulated the Ca uptake as well as CA secretion in bovine adrenal glands (Table 2). As shown in Fig. 4 and Table 2, SGTX (20 μM) or mecamylamine (0.1 mM) significantly reduced the stimulation of Ca uptake and CA secretion induced by DMPP (0.3 mM) and ACh (0.3 mM).

There is some evidence to suggest the action of nicotinic agonists on the adrenal cortex: a stimulatory effect of nicotinic agonists on corticoid secretion (15) and steroidogenesis (16) in adrenal cortex, and an increase in $^{45}$Ca uptake by perfused cat adrenal cortex during the ACh perfusion (6). Thus adrenal glands preloaded with $^{45}$Ca in the absence and presence of DMPP were washed with Ca free Locke's solution for 48 min and the radioactivity in the medulla and cortex was measured. The radioactivity ($^{45}$Ca) remaining in the medulla and cortex from the $^{45}$Ca preloaded bovine adrenal glands was $4538±639$ (N=3) and $3674±272$ (N=3) dpm /g wet weight, respectively. In the DMPP (0.3 mM)- and ACh (0.3 mM)-stimulated adrenal glands, the remaining $^{45}$Ca in the medulla was $9806±885$ (N=3) and $8142±840$ (N=3) dpm/g wet weight, respectively. Thus there was a significant (P<0.01) increase (116% and 79% respectively) in the $^{45}$Ca uptake by the DMPP- and ACh- stimulated medulla as compared to the nonstimulated medulla. In contrast, there was no significant alteration in cortical $^{45}$Ca uptake in the agonists-stimulated adrenal glands (the remaining $^{45}$Ca in the adrenal cortex: DMPP stimulation, 4316±309 dpm/g wet weight, N=3; ACh stimulation, 3141±501 dpm/g wet weight, N=3).

**DISCUSSION**

We found that nicotinic receptor agonists increase the uptake and efflux of $^{45}$Ca in perfused bovine adrenal glands. The stimulation of Ca flux by nicotinic agonists which closely correlated with the secretory response of CA was significantly inhibited by nicotinic receptor antagonists such as SGTX and mecamylamine.

A critical role of Ca in the process of CA secretion from adrenal medullary cells has been well reviewed (1, 5, 6). Secretion of CA from adrenal glands elicited by ACh, high potassium or ionophores is mediated by the influx of Ca from the extracellular medium (2-4, 10). In agreement with these previous reports, we have demonstrated the significantly increased uptake of $^{45}$Ca during the perfusion of bovine adrenal glands with DMPP and ACh. Since the enhanced uptake of $^{45}$Ca by nicotinic agonists preceded the stimulation of CA secretion, the entry of Ca into the medullary cells seems to initiate the secretory response to the agonists (5, 8, 9).

The apparent decline in the stimulation of $^{45}$Ca uptake which was observed 3 to 15 min following the perfusion with nicotinic agonists may be partially due to the increased efflux of $^{45}$Ca by these agonists as demonstrated in the $^{45}$Ca preloaded adrenal glands. Douglas and Poisner (6) found an increase in the $^{45}$Ca uptake by the perfused cat adrenal cortex during ACh perfusion. However, in the present study, nicotinic agonists did not significantly alter the $^{46}$Ca uptake by the bovine adrenal cortex. Since there was a significant increase in the medullary uptake of $^{45}$Ca by DMPP- and ACh-stimulated adrenal glands without a change in the $^{45}$Ca uptake by the cortex, the increased uptake of $^{45}$Ca by nicotinic agonists in perfused bovine adrenal glands may reflect an alteration...
in the Ca uptake by adrenal medulla and not by the cortex. Similarly, Borowitz (11) found that there was a significant increase in the \(^{45}\text{Ca}\) content of bovine adrenal medulla perfused with the Locke's solution containing \(^{45}\text{Ca}\) and ACh as compared to the nonstimulated medulla. Since SGTX and mecamylamine significantly reduced the increased Ca uptake as well as CA secretion by DMPP and ACh in perfused bovine adrenal glands, these data suggest that the increased entry of Ca into the medullary chromaffin cells by the agonists is mediated by their stimulation of the nicotinic receptors.

Nicotinic agonists caused a significant increase in the \(^{45}\text{Ca}\) efflux from bovine adrenal glands preloaded with \(^{45}\text{Ca}\). A stimulation of \(^{45}\text{Ca}\) efflux by DMPP and ACh showed a time course similar to an increase in the CA secretion and was completely inhibited by the perfusion with Ca free Locke's solution. During the perfusion of adrenal glands with Ca free Locke's solution containing ACh, an application of high Ca (22 mM, 4 min) restored the stimulation of \(^{45}\text{Ca}\) efflux and CA secretion by the agonists. The Ca-induced efflux of \(^{45}\text{Ca}\) in perfused cat adrenal glands was described by Rubin et al. (7). Therefore the enhanced efflux of \(^{45}\text{Ca}\) by nicotinic agonists in bovine adrenal glands seems to be associated with the CA secretion. Whether the increased efflux of \(^{45}\text{Ca}\) by nicotinic agonists is related to the chromaffin granules or to the other cellular sites is not clear in the present study. Since Ca is localized in high concentrations in the CA containing granules (17) and since the Ca bound to isolated granule membranes is exchangeable with Ca in the medium (18), the large efflux of \(^{45}\text{Ca}\) accompanying CA secretion by nicotinic agonists could be due to the release of labeled Ca from the granules. Alternately, the \(^{45}\text{Ca}\) efflux stimulated by nicotinic agonists may be associated with the release of \(^{45}\text{Ca}\) bound to the superficial membranes since ACh facilitates the permeability of chromaffin cell membranes by displacing a critical Ca fraction bound to the cell membranes (19). SGTX and mecamylamine in the concentrations which abolished the \(^{45}\text{Ca}\) uptake and CA secretion by nicotinic agonists, significantly decreased the DMPP- or ACh-induced \(^{45}\text{Ca}\) efflux in the \(^{45}\text{Ca}\) preloaded bovine adrenal glands. Thus our data have demonstrated that nicotinic agonists stimulate the uptake and efflux of \(^{45}\text{Ca}\) as well as CA secretion in perfused bovine adrenal glands. Nicotinic antagonists may inhibit the stimulatory response of Ca flux to nicotinic agonists by an action on the nicotinic receptors of medullary chromaffin cells and the subsequent CA secretion. Since Ca exchange between the perfused cat adrenal gland and the perfusate was markedly depressed by tetracaine and Mg\(^{2+}\) (7) and since there was no significant increase in total Ca content in the ACh-stimulated bovine adrenal medulla (11), adrenal CA secretion by nicotinic agonists seems to be closely associated with their stimulation of Ca exchangeability as suggested also in the present study.

Although the concentration (0.3 mM) of ACh and DMPP used in the present study was relatively high as compared to that used in the \textit{in vitro} pharmacological experiment with isolated other organs (13), we did not find a significant increase in the adrenal CA secretion under the experimental condition of low agonist concentration (1 \(\mu\)M). To stimulate CA secretion in perfused adrenal glands, similar high concentrations (0.1–1 mM) of ACh were in general used also by previous workers (7, 8, 20). Since adrenal glands were constantly superfused in a retrograde manner through the adrenal central vein, this type of experiment seems to differ from the \textit{in vitro} pharmacological experiment in which relatively low concentrations of agonists produce a biological
response (13) and thus to require a high concentration of agonist for the significant CA secretion.

In conclusion, the present study has shown that nicotinic agonists-induced secretion of CA which is mediated by a stimulation of the nicotinic receptors may be related to an enhancement of uptake and efflux of Ca in perfused bovine adrenal glands and that nicotinic antagonists may inhibit the stimulated flux of Ca by the agonists and the subsequent CA secretion.

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