Contribution of intra- and extracellular Ca^{2+} to noradrenaline exocytosis induced by ouabain and monensin from guinea-pig vas deferens

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1 Contributions of intra- and extracellular Ca^{2+} to noradrenaline (NA) release evoked by increasing intracellular Na^+ concentrations (ouabain plus monensin) from adrenergic nerves of guinea-pig vas deferens were evaluated under conditions eliminating carrier-mediated NA release (with 100 μM cocaine).

2 Ouabain (100 μM) plus monensin (10 μM), unlike 100 mM KCl, produced a marked NA release which was unchanged by Ca^{2+} -removal.

3 In normal solution but not in Ca^{2+} -free solution, the release of NA evoked by ouabain plus monensin was reduced by adenosine, clonidine and neuropeptide Y, and by Ca^{2+} -channel blockers such as ω -conotoxin GVIA and nifedipine. The release of NA was also decreased by cromakalim in a glibenclamide-sensitive fashion.

4 In contrast, in the absence but not in the presence of Ca^{2+} , the drug-evoked NA release was inhibited by mitochondrial inhibitors (carbonylcyanide-*m*-chlorophenylhydrazone and oligomycin) and further by immobilizers of intracellular Ca^{2+} (TMB-8 and BAPTA-AM) and calmodulin antagonists (W-7 and trifluoperazine).

5 These findings suggest that the release of NA evoked by elevation of $[\text{Na}^+]_i$ from adrenergic nerves in the presence and absence of Ca^{2+} involves, in part, exocytotic processes which are triggered by depolarization-induced Ca^{2+} influx and by utilization of Ca^{2+} from intracellular Ca^{2+} store sites such as mitochondria, respectively.

Keywords: Noradrenaline release; extra- and intracellular Ca^{2+} ; ouabain plus monensin; neuromodulators; Ca^{2+} -antagonists; guinea-pig vas deferens

Introduction

Ouabain, a cardiac glycoside, is generally used to inhibit $\text{Na}^+\text{-K}^+$ ATPase and subsequently, to block the Na^+ pump on the cell membrane (Aker, 1977; Török, 1989). There is accumulated evidence for a role of ouabain as a trigger in adrenergic (Paton, 1973; Nakazato *et al.*, 1978) or cholinergic (Paton *et al.*, 1971; Vizi, 1972, 1978) neurotransmission.

According to Nakazato and co-workers (1978), the increase in the noradrenaline (NA) output after exposure to ouabain was antagonized by Ca^{2+} removal from the medium. Recent studies, however, revealed that NA release evoked by ouabain but not by electrical stimulation or KCl, from adrenergic nerves of guinea-pig vas deferens (Katsuragi *et al.*, 1988) and heart (Kranzhöfer *et al.*, 1991), from rat tail artery (Palaty, 1981) and neocortical slices (Schoffeleer *et al.*, 1988) was virtually unaffected or even enhanced in Ca^{2+} -free medium. Therefore, the mechanism underlying release of transmitters evoked by increasing $[\text{Na}^+]_i$ is still unknown. It has been hypothesized that the positive inotropic action (Orrego, 1984) and catecholamine release from bovine chromaffin cells (Török & Powis, 1990) evoked by cardiac glycosides may be caused by a $\text{Na}^+\text{-Ca}^{2+}$ exchange mechanism across the cell membrane (Orrego, 1984). However, the contribution of mitochondrial Ca^{2+} , which might be released by reversal of $\text{Na}^+\text{-Ca}^{2+}$ exchange between cytosol and mitochondria on raising $[\text{Na}^+]_i$, to the release of neurotransmitters remains to be clarified.

In recent studies (Katsuragi *et al.*, 1988), it has been shown that the application of monensin, a Na^+ ionophore (Pressman & Fahim, 1982), in combination with ouabain, produced a quick and marked increase in intracellular Na^+

concentration ($[\text{Na}^+]_i$) which results in massive release of NA from adrenergic nerves.

Accordingly, in the present study, the contribution of intra- and extracellular Ca^{2+} to NA release evoked from guinea-pig vas deferens by elevating $[\text{Na}^+]_i$ with ouabain plus monensin, was extensively evaluated in the presence and absence of Ca^{2+} by using presynaptic neuromodulators, such as clonidine and ω -conotoxin GVIA, intracellular Ca^{2+} -chelators, mitochondrial inhibitors and calmodulin antagonists.

Methods

Measurement of NA release

Male guinea-pigs (250–400 g) were stunned and bled. Both vasa deferentia were removed and dissected free of fascia and vascular tissue. Preparations were trimmed to 15 mm in length. These segments were suspended with an initial load of 0.7 g in an organ bath filled with 1 ml of Krebs solution (37°C) of the following composition (mM): NaCl 122, KCl 5.2, CaCl_2 2.4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, NaHCO_3 25.6, D-glucose 11, EDTA 0.03 and ascorbic acid 0.1. The bathing medium was constantly bubbled with 95% O_2 + 5% CO_2 . Then, pargyline (10 μM ; a monoamine oxidase inhibitor), U-0521 (50 μM ; a catechol-*O*-methyltransferase inhibitor) and cocaine (100 μM ; an amine uptake inhibitor) were added to the bath and kept there throughout the experiment to measure NA as the metabolically unchanged form. The addition of 100 μM cocaine also inhibits carrier-mediated $\text{Na}^+\text{-NA}$ co-transport (Schömig *et al.*, 1988; Adam-Vizi, 1992). Thirty minutes after suspending the tissue, secretagogues such as ouabain and monensin, were introduced into the bath for the test period

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of 40 min. Use of organic solvents such as ethanol was limited to 0.05% of the bathing medium without any solvent effect. The Ca^{2+} -free solution was made by removal of Ca^{2+} from Krebs solution and by the addition of 0.5 mM EGTA.

Medium (100 μl) was collected from the bath prior to, 0 (control), 10, 20, 30 and 40 min after administration of drugs such as ouabain. Following each sampling, fresh Krebs solution (100 μl) of the same composition as the experimental medium was added to the bath. Endogenous NA in the sample medium (100 μl) was adsorbed onto 50 mg of alumina (N-Super 1, ICN Biochemicals, Cleveland, OH, U.S.A.) in a centrifuge tube with a filter (0.22 μm) and centrifuged for 1 min with a microcentrifuge (6,400 r.p.m.). NA adsorbed on alumina was eluted with 0.2 N HCl (100 μl) through centrifugation as described above. At the end of the experiment, tissue wet weight was measured after blotting with filter paper. Amounts of NA in the aliquots (10 μl) were determined with a high performance liquid chromatography electrochemical detector system (h.p.l.c.-e.c.d., Yanagimoto MFG Co., Kyoto, Japan) equipped with a reversed phase column (Yanapak, ODS-T) as described previously (Katsuragi *et al.*, 1988; 1991). Briefly, the mobile phase consisted of 2.5 mM sodium-1-octane sulphonate, 10 μM EDTA:2Na and 163 ml of ethanol in 1000 ml of 0.1 M KH_2PO_4 . The phosphate buffer was adjusted to pH 3.3 with phosphoric acid. Flow rate was 1.0 ml min^{-1} (25°C). Finally, because an amine-free medium (100 μl) was added to the bathing medium (900 μl) after sampling, experimental values of NA were compensated for this and expressed as $\mu\text{g g}^{-1}$ wet weight of tissue or % of control.

Test inhibitors were applied in effective concentrations without affecting the basal release of NA.

Drugs

The drugs used were ouabain (E. Merk, Darmstadt, Germany); monensin (gift from Eli Lilly, Indianapolis, IN, U.S.A.); U-0521 (3,4-dihydroxy-2-methyl propiophenone; gift from Upjohn, Kalamazoo, MI, U.S.A.); cocaine hydrochloride (Takeda, Osaka, Japan); pargyline hydrochloride (Nacalai, Kyoto, Japan); adenosine hemisulphate, clonidine hydrochloride, nifedipine, CCCP (carbonyl cyanide-*m*-chlorophenylhydrazone), oligomycin, TMB-8 (3,4,5-trimethoxy benzoate 8-(*N,N*-diethyl amino) octyl ester), W-7 (N-(6-amino hexyl)-1-naphthalene sulphonamide), trifluoperazine (TFP; Sigma, St. Louis, MO, U.S.A.); neuropeptide Y, ω -conotoxin GVIA (Peptide Institute, Minoh, Japan); BAPTA-AM (1,2-bis(O-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid aminoethoxy) (Dojin, Kumamoto, Japan); cromakalim (gift from Beecham, Harlow, U.K.) and glibenclamide (gift from Yamanouchi, Tokyo, Japan).

Statistical analysis

Differences between two means and between multiple means were tested for statistical significance by Student's *t* test and by one way analysis of variance (ANOVA) followed by Dunnett's test, respectively. A value of $P < 0.05$ was considered to be significant.

Results

Effects of external Ca^{2+} on NA releases evoked by ouabain, monensin and KCl

Efflux of NA from adrenergic nerves of guinea-pig vas deferens was measured by sampling every 10 min for up to 40 min after administration of test drugs in the presence and absence of Ca^{2+} in the external medium. Ouabain (100 μM) and monensin (10 μM) in the presence of Ca^{2+} increased the total release of NA during 40 min by approximately 10 and 6 fold, respectively, from a basal efflux of $0.67 \pm 0.05 \mu\text{g g}^{-1}$

wet weight ($n = 8$). The release of NA evoked by ouabain was further enhanced by concurrent administration of monensin. The NA release induced by ouabain plus monensin was unchanged by Ca^{2+} removal from the medium except for the first 10 min period after adding the secretagogues. Similarly, the basal release of NA was virtually unaffected by Ca^{2+} -free medium (Figure 1a). On the other hand, 100 mM KCl induced a much lower NA release of about 20% compared to that by ouabain plus monensin. The KCl-evoked NA release was largely inhibited by removal of Ca^{2+} (Figure 1b).

Figure 1 Effects of ouabain and/or monensin (a) and high KCl (b) on noradrenaline release from guinea-pig vas deferens in the presence and absence of Ca^{2+} . (a) (○) Basal release ($n = 8$); (□) basal release without Ca^{2+} ($n = 6$); (●), 100 μM ouabain ($n = 9$); (■) 10 μM monensin ($n = 5$); (▲) ouabain plus monensin ($n = 5$); (Δ) ouabain plus monensin without Ca^{2+} ($n = 5$). (b) (○) Basal release ($n = 8$); (▲) 100 mM KCl ($n = 5$); (Δ) 100 mM KCl without Ca^{2+} ($n = 4$). These experiments were carried out in the presence of Ca^{2+} , except for the experiment shown with (Δ). Sampling at 0 min was actually carried out 5 min before adding secretagogues. Values from each media sample which were collected cumulatively every 10 min up to 40 min are expressed as μg of noradrenaline per gram wet weight of tissue (mean $\pm$ s.e.mean). * $P < 0.05$; ** $P < 0.01$ from control with Ca^{2+} .

[Ca²⁺]_o-dependent effects of drugs on NA release evoked by ouabain plus monensin

The total NA released in the 40 min after administration of ouabain and monensin in normal medium was significantly inhibited by approximately 27%, 26% and 31% by addition of adenosine (30 μ M), clonidine (30 μ M) and neuropeptide Y (3 μ M), respectively (Figure 2a).

In Ca²⁺-free medium, inhibition of the evoked NA release by these drugs was no longer detectable (Figure 2b). ω -Conotoxin GVIA and nifedipine at a concentration of 0.3 μ M also reduced the evoked NA release to 66% and 69%, respectively, in the presence of Ca²⁺, although nifedipine, unlike ω -conotoxin GVIA, tended to decrease the release even in Ca²⁺-free conditions. Furthermore, cromakalim

(1 μ M) reduced the evoked NA release in normal medium. This reduction by cromakalim did not occur in the presence of glibenclamide (1 μ M), a K⁺-channel blocker, or in the absence of Ca²⁺ (Figure 3). These findings, thus, show that the reduction by these test drugs of the NA release evoked by ouabain plus monensin in the presence, but not absence, of Ca²⁺ can be attributed to inhibition of Ca²⁺-influx from the external medium.

None of the test drugs used here affected the basal NA release (data not shown).

Effects of drugs on NA release evoked by ouabain plus monensin in Ca²⁺-free medium

Following exposure to 10 μ M CCCP or 30 μ M oligomycin (mitochondrial inhibitors), the NA release evoked by ouabain plus monensin from the adrenergic nerves was reduced by approximately 30% in Ca²⁺-free medium, but was virtually unaffected in the presence of Ca²⁺ (Figure 4). The NA release caused by increasing [Na⁺]_i with ouabain plus monensin under Ca²⁺-free conditions was inhibited by about 38%

Figure 2 Effects of adenosine (Aden), clonidine (Clon) and neuropeptide Y (NPY) on noradrenaline release evoked by ouabain plus monensin in the presence (a) and absence (b) of Ca²⁺. Cumulative release of noradrenaline over 40 min after concurrent administration of ouabain (100 μ M) and monensin (10 μ M) is expressed as % (mean $\pm$ s.e.mean) of the corresponding control; (a) $9.01 \pm 1.27 \mu$ g g⁻¹ wet weight 40 min⁻¹, $n = 5$; (b) $8.49 \pm 1.32 \mu$ g g⁻¹ wet weight, 40 min⁻¹, $n = 5$. A test drug was added to the bath 10 min before introduction of ouabain and monensin and remained during the experiment. Number of experiments is shown in each column. * $P < 0.05$ from control.

Figure 3 Effects of ω -conotoxin GVIA (ω -Con), nifedipine (Nif) and cromakalim (Crom) on noradrenaline release evoked by ouabain plus monensin in the presence (a) and absence (b) of Ca²⁺. For details, see Figure 2. Glibenclamide was added 5 min before cromakalim. * $P < 0.05$ from control.

Figure 4 Effects of carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) and oligomycin (Oligo) on the noradrenaline release evoked by ouabain plus monensin in the presence and absence of Ca^{2+} . For details, see Figure 2. Test antagonists were added to the bath 20 min before administration of ouabain plus monensin. * $P < 0.05$ from control.

and 31%, respectively, by 100 μM TMB-8, a blocker of intracellular Ca^{2+} mobilization, and 10 μM BAPTA-AM, an intracellular Ca^{2+} -chelator, suggesting that the NA release evoked by ouabain plus monensin, even in Ca^{2+} -free medium, may be elicited, in part, by using intracellular free Ca^{2+} (Figure 5). Finally, the effect of calmodulin antagonists on the evoked release of NA was assessed in the Ca^{2+} -free medium. This NA release was reduced by approximately 33% and 43%, respectively, following exposure to W-7 and TFP at 30 μM (Figure 5).

These antagonists did not significantly alter the basal release of NA from the tissue as illustrated in Table 1.

Discussion

We have found that ouabain plus monensin produced a marked and long-lasting release of NA from adrenergic nerves of the guinea-pig vas deferens, even in Ca^{2+} -free medium. The results described in this paper provide evidence that the mechanisms involved in this release of NA differ in Ca^{2+} -containing and Ca^{2+} -free medium.

The combination of inhibition of the Na^+ -pump by ouabain and increase in Na^+ permeability by monensin (a Na^+ ionophore) will result in an accumulation of Na^+ and loss of K^+ in the axons. The reduction in the K^+ gradient will result in depolarization, which, in the presence of extracellular Ca^{2+} could trigger Ca^{2+} entry through voltage-sensitive Ca^{2+} channels and release of NA. However, the release of NA evoked by 100 mM KCl, which was Ca^{2+} -dependent, was small in comparison, suggesting that the increase in intracellular Na is likely to play an additional role in stimulating release. The release of NA evoked by ouabain plus monensin (with or without Ca^{2+}) increased with time during the first 30 min, and then stopped. This could be due to depletion of releasable NA from the nerve endings, since the NA content of the vas deferens is approximately 10 $\mu\text{g g}^{-1}$ wet weight (unpublished data).

In the presence of Ca^{2+} , but not in its absence, the release of NA evoked by ouabain plus monensin was reduced by the

Figure 5 Effects of TMB-8, BAPTA/AM, TFP and W-7 on noradrenaline release evoked by ouabain plus monensin in the absence of Ca^{2+} . For details, see Figure 4. Protocol of sampling was identical with that in Figure 2b. * $P < 0.05$; ** $P < 0.01$ from control. For abbreviations, see text.

Table 1 Effects of antagonists on basal noradrenaline release

Treatments	(μM)	(n)	Released noradrenaline		P
			(μg g ⁻¹ wet wt. 15 min ⁻¹)	mean ± s.e.mean	
No treatment		(8)	0.38 ± 0.03		
Oligomycin	30	(5)	0.44 ± 0.08		NS
TFP	30	(8)	0.38 ± 0.04		NS
TMB-8	100	(6)	0.42 ± 0.06		NS
BAPTA/AM	10	(5)	0.40 ± 0.05		NS

Antagonists were added to the bath 15 min before sampling of basal noradrenaline release. NS, not significantly different from control. For abbreviations, see text.

addition of adenosine, clonidine and neuropeptide Y. The fact that these agents did not have an effect in the absence of Ca^{2+} supports the suggestion that they could be blocking NA release by reducing Ca^{2+} entry (adenosine: Hedqvist & Fredholm, 1976; Su, 1983; clonidine: Starke, 1977; neuropeptide Y: Lundberg & Stjärne, 1984). This view can be further supported by the finding that the evoked NA release was reduced by voltage-gated Ca^{2+} channel blockers such as ω -conotoxin GVIA and nifedipine, but again only in the presence of Ca^{2+} . It has been accepted that activation of N-type Ca^{2+} channels, which are sensitive to ω -conotoxin GVIA, plays an important role in the depolarization-dependent NA release from sympathetic neurones (Hirning *et al.*, 1988). Moreover, studies in the gastrointestinal tract provide evidence that the electrically-evoked ACh release from both the guinea-pig isolated colon (Lecchini *et al.*, 1991) and ileum (Katsuragi *et al.*, 1990) was inhibited by nifedipine but was enhanced by Bay K 8644. According to Artalejo & Garcíá (1986), catecholamine release from cat adrenal glands evoked by ouabain, but not by A-23187 (a Ca^{2+} -ionophore), was strongly enhanced by Bay K 8644, indicating that catecholamine release induced by ouabain results from, in part, L-type Ca^{2+} channel activation via cell depolarization (Vizi, 1978). These findings, therefore, are highly consistent with our results.

In the present work with normal Krebs solution, cromakalim, an ATP-sensitive- K^+ channel opener, produced a decrease of evoked NA release, which was reversed by glibenclamide, an ATP sensitive- K^+ channel blocker (Nielsen-Kudsk *et al.*, 1990; Satoh *et al.*, 1991). This suggests that, under these conditions, the K^+ -channel opener cromakalim can still hyperpolarize the nerve terminal membrane, thus reducing Ca^{2+} entry through voltage sensitive Ca^{2+} channels.

In contrast to the inhibitory action of these neuromod-

ulators, a mitochondrial inhibitor, oligomycin, inhibited the evoked NA release in the absence, but not in the presence, of Ca²⁺ in the medium. Similarly, CCCP, an uncoupler of mitochondria, was able to reduce the evoked NA release only in Ca²⁺-free conditions. BAPTA/AM is a permeant derivative of BAPTA for binding cytosolic free Ca²⁺. Thus, this chelator can inhibit intracellular Ca²⁺-requiring processes such as synaptic transmission (Niesen *et al.*, 1991) and secretion from salivary glands (Mills *et al.*, 1991). TMB-8 is known as an inhibitor of Ca²⁺ release from intracellular stores, i.e. a Ca²⁺ immobilizer, and prevents Ca²⁺-influx across the cell membrane (Kojima *et al.*, 1985). In our study, BAPTA/AM and TMB-8 also diminished NA release evoked by ouabain plus monensin in Ca²⁺-free medium. In further studies, W-7 and TFP, Ca²⁺/calmodulin antagonists, diminished the release of NA evoked by ouabain plus monensin in the Ca²⁺-free medium. Therefore, a calmodulin-dependent process may be involved in NA release triggered by an increase of [Na⁺]_i in Ca²⁺-free conditions.

These results suggest that increase of [Na⁺]_i may elicit reversed Na⁺-Ca²⁺ exchange between cytosolic Na⁺ and mitochondrial Ca²⁺ (Sandoval, 1981; Crompton, 1985). This mechanism must be of great importance in the Ca²⁺-free solutions where Ca²⁺ entry cannot occur, but does not seem to occur in Ca²⁺-containing solutions. This clearly suggests that the two conditions are rather different. It is possible that a greater accumulation of Na⁺ and loss of K⁺ may occur when the Na pump is blocked in Ca²⁺-free solutions, than when Ca²⁺ is present, as has been seen in smooth muscle (Aickin *et al.*, 1987). The fact that cromakalim can reduce NA secretion evoked by ouabain and monensin in Ca²⁺-containing solutions strongly suggests that the K⁺ equilibrium potential is still positive to the resting potential, and

the axon terminals must contain significant amounts of K⁺. In Ca²⁺-containing solutions although part of the release is triggered by Ca entry, and can be reduced by the test drugs, it is noticeable that even at high concentrations the test drugs only reduced the release by 30–35%, and never abolished it. This suggests that another, unknown mechanism of release is involved. It is possible that Ca²⁺-loss from mitochondria via Na⁺-Ca²⁺ exchange reduces oxidative ATP synthesis in this organelle (Cox & Matlib, 1993) and the consequent reduction of ATP at catecholamine store sites might trigger release of bound NA from the nerve terminals (Trifarò & Cubeddu, 1979).

In conclusion the release of NA caused by ouabain plus monensin seems to be complicated. In normal Ca²⁺ medium, NA release evoked by accumulation of [Na⁺]_i seems to involve an exocytotic release process, which is partly due to the depolarization-induced Ca²⁺ influx from the outer medium. However, in Ca²⁺-free medium when intracellular Na⁺ is probably higher, the NA release could be triggered by an exocytotic process by using intracellular Ca²⁺ derived from Ca²⁺ store sites such as mitochondria, e.g. via the Na⁺-Ca²⁺ exchange transport mechanisms. In addition to these Ca²⁺-requiring processes, there may be further unknown mechanisms involved in the massive release of NA following accumulation of [Na⁺]_i.

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