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Axoplasmic Transport of [^{3}H]Ouabain Binding Sites and Catecholamine Secretion from an Adrenergic Nerve Trunk

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SUMMARY

The presence of a functional Na^+/Ca^{2+} exchange system was explored in the ligated cat hypogastric nerve, a preparation that has been proposed as a model of giant noradrenergic nerve terminal free of effector cells. The rationale for this study was to monitor noradrenaline secretion from the ligated cat hypogastric nerve promoted by the increase in intracellular Ca^{2+} levels after ouabain blockade of Na^+,K^+-ATPase molecules present in the plasma membrane of the ligated cat hypogastric nerve. Such an increase in intracellular Ca^{2+} levels is achieved by activation, in "reverse mode," of the Na^+/Ca^{2+} exchange system. In the present study, [^{3}H]ouabain binding sites were identified on crude preparations of hypogastric nerve membranes. A single, high affinity (K_d around 10 nM), binding site was observed in both ligated and nonligated nerves. The number of binding sites increased with the time of ligation, reaching a peak of about 1 pmol/mg of protein 48 hr after ligation. Blockade of these binding sites by ouabain induced a dose-dependent, Ca^{2+}-dependent release of noradrenaline, with an ED_{50} around 50 μM. The maximum release amounted to 9% of the total noradrenaline content in the cells. As would be expected for ouabain-induced noradrenaline secretion mediated by a Na^+/Ca^{2+} exchange system working in reverse mode, the effect of ouabain was dependent upon the presence of Na^+ in the incubation medium, reaching a plateau at an extracellular Na^+ concentration of 100 mM. Calcium uptake after Ca^{2+} reintroduction in ouabain-treated nerves increased with time of ligation, suggesting the incorporation of Na^+/Ca^{2+} exchange carrier molecules into the axolemma of hypogastric nerves. The similarity between ouabain-induced noradrenaline secretion from the ligated cat hypogastric nerve and from other adrenergic systems strongly supports the idea that the ligated cat hypogastric nerve is equipped with a functional Na^+/Ca^{2+} exchange system that would contribute to the regulation of intracellular Ca^{2+} levels. Furthermore, these data, together with previously published reports, fully characterize, from a biochemical point of view, the ligated hypogastric nerve as a model of giant noradrenergic nerve terminal free of effector cells.

Maintenance of large Ca^{2+} electrochemical gradients in most living cells depends on the presence of Ca^{2+} extrusion mechanisms located on the plasma membrane (1). Part of this Ca^{2+} efflux seems to be dependent on $[Na^+]_o$ (2, 3). Such a Na^+/Ca^{2+} exchange mechanism has been described in a wide variety of excitable tissues, including squid giant axons (4), heart (5), and adrenergic tissues (6–8). One of the characteristics of the Na^+/Ca^{2+} exchange system is that, in response to changes in Na^+ electrochemical gradient, it can work in "reverse mode," introducing Ca^{2+} into the cell (9). This property has been widely used in secretory systems, like sympathetic nerve terminals (7), perfused adrenal glands (6, 8, 10), or isolated bovine chromaffin cells (11), to study the Na^+/Ca^{2+} exchange system. The general approach has been to determine neurotransmitter or hormone secretion as an index of the increase in $[Ca^{2+}]_i$ obtained through the Na^+/Ca^{2+} exchange system, working in reverse mode, that follows ouabain administration.

The ligated cat hypogastric nerve shares many of the properties of sympathetic nerve terminals. It releases CA in response to different stimuli (like high K^+ and electrical stimulation) that induce CA release from sympathetic nerve terminals (8). It has cholinoceptors (12) and Na^+ and Ca^{2+} channels (13, 14), transports and accumulates calmodulin (15), and can take up NA by an uptake system that is sensitive to cocaine and imipramine (16). However, more properties that are characteristic of sympathetic nerve terminals should be shown in the ligated cat hypogastric nerve before it can be fully accepted as an adrenergic nerve terminal free of effector cells. Two of these properties concern the presence of functionally active Na^+,K^+-ATPase and a Na^+/Ca^{2+} exchange mechanism that would contribute to keep resting $[Ca^{2+}]_i$ in this tissue.

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ABBREVIATIONS: $[Na^+]_o$, extracellular sodium concentration; $[Ca^{2+}]_i$, intracellular free calcium concentration; NA, noradrenaline; CA, catecholamines; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K-B, Krebs-bicarbonate.

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The aim of this work was to establish the presence of a Na^+/Ca^{2+} exchange mechanism and Na^+,K^+-ATPase, which, together with previously published data (8, 12–16), would fully characterize, from a biochemical point of view, the ligated cat hypogastric nerve as a giant noradrenergic nerve terminal free of effector cells. We approached this problem by studying the characteristics of ouabain-induced NA secretion from 48-hr ligated cat hypogastric nerve. We found that there is an axoplasmic transport of [^{3}H]ouabain binding sites in the cat hypogastric nerve and that blockade of those sites by ouabain induces a Ca^{2+}-dependent NA secretion whose magnitude is related to $[Na^+]_o$. In addition, the amount of Ca^{2+} taken up by the nerve increases with time of ligation, suggesting an incorporation of Na^+/Ca^{2+} exchange carriers in the hypogastric neurons axolemma. These results strongly support the presence, in the ligated cat hypogastric nerve, of a Na^+/Ca^{2+} exchange mechanism that could contribute to the regulation of $[Ca^{2+}]_i$ in this tissue.

Materials and Methods

Ligation of nerves. Cats of either sex, weighing 2.5 to 4 kg, were anesthetized with ether. The abdomen was opened by a midline incision, under aseptic conditions, and the hypogastric nerves were tied with a silk thread about 2.5 cm from the inferior mesenteric ganglia. A second ligature was placed 3 mm distal to the previous one (12). After the abdomen was closed, penicillin G (600,000 IU/kg) was injected intramuscularly and cats were allowed to recover, having food and water *ad libitum*. At the appropriate time, animals were anesthetized again (induction with ether and anesthesia with 60 mg/kg chloralose, intravenously), the abdomen was reopened, and the proximal parts of the nerves (1 cm) were carefully dissected under magnification, removed, and used for the experiments.

Membrane preparation. Hypogastric nerves (12 for each time) were ligated at different times, carefully cleaned from connective tissue under magnification, pooled, and homogenized in 5 ml of buffer I (150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 50 mM Tris·HCl, pH 7.4). The homogenate was centrifuged at 800 × *g* for 5 min, and the supernatant was used for all binding assays. Protein was measured as described by Lowry *et al.* (17).

[^{3}H]Ouabain binding. Membranes (50 μg of protein) were incubated (90 min, 37°) with [^{3}H]ouabain (1–50 nM; specific activity, 20 Ci/mmol) in buffer I containing ATP (1.25 mM) (final volume, 1 ml). The incubation was terminated by immersing the tubes in a 4° bath for 10 min. No appreciable loss of bound radioactivity was observed during this period of time. Nonspecific binding, measured either in the absence of ATP or in the presence of high doses of unlabeled ouabain (100 μM), was always <10% of total binding. Membrane-bound [^{3}H]ouabain was collected with a M-24 cell harvester (Brandel, Gaithersburg, MD), using Whatman glass fiber filters (GF/C). The filters were washed three times with 5 ml of buffer I (4°), dried (30 min, room temperature), and then placed into a vial containing 10 ml of scintillation liquid (HP Ready-Solv; Beckman, Fullerton, CA). Radioactivity was measured in a Beckman LS2800 scintillation counter. Saturation curves were analyzed using the nonlinear curve-fitting program LIGAND (18).

Labeling of the nerves. Segments (1 cm) from ligated hypogastric nerves proximal to the ligature were cleaned of connective tissue and placed into the incubation chamber, as previously described (12). Nerves were incubated for 10 min in 1 ml of K-B (144 mM NaCl, 2.5 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM KCl, 25 mM $NaCO_3H$, 11 mM glucose, 15 mM HEPES, pH 7.4). The solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2. After initial equilibration, the nerves were transferred into tubes containing 1 ml of K-B and 0.1% collagenase (type A; Boehringer-Mannheim, Indianapolis, IN), for 30 min (3 × 10 min). The collagenase treatment has been shown to disrupt a diffusion barrier for polar molecules present in the ligated hypogastric nerve (16). After collagenase treatment, nerve trunks were incubated for 30 min in K-B containing 1 μCi of [^{3}H]NA (specific activity, 13 Ci/mmol). After 60 min (12 × 5 min) of washing in K-B, nerves were transferred to Ca^{2+}-free K-B containing the desired amount of ouabain. This point was considered time 0 (see Fig. 3).

[^{3}H]NA secretion. Ligated nerves were incubated with ouabain in Ca^{2+}-free medium for 60 min (12 × 5 min), to allow the molecule to bind to the receptor. We chose this time because association time-course studies in other neural tissues indicated that at this time about 90% of ouabain is bound to its receptor (19). After this 60-min period without Ca^{2+} and in the presence of ouabain, Ca^{2+} (2.5 mM) was reintroduced into the incubation medium. The 30-min (6 × 5 min) period after Ca^{2+} reintroduction, in the presence of ouabain, was considered the period of stimulation. Twenty minutes after ouabain removal, nerves were stimulated with high K^+ (125 mM) for 5 min, to assess the preparation functionality. A typical experiment can be observed in Fig. 3. When used, different Na^+ concentrations in the extracellular medium were achieved by decreasing the NaCl content. Isosmolarity was obtained by equiosmolar substitution of NaCl by sucrose.

Release is expressed as fractional release, i.e., the amount of radioactivity released during the incubation period divided by the total amount of radioactivity calculated to be present in the nerve at the beginning of that specific incubation period (×100).

The statistical significance of the differences between means was determined using Student's *t* test for grouped data.

$^{45}Ca^{2+}$ uptake. Hypogastric nerves were incubated with ouabain (1 mM), in Ca^{2+}-free medium for 60 min (12 × 5 min). After this 60-min period without Ca^{2+} and in the presence of ouabain, the nerves were transferred to a tube containing 1 ml of K-B (2.5 mM $CaCl_2$) with 20 μCi of $^{45}CaCl_2$ (specific activity, 20 Ci/mg; 0.62 mg/ml; Amersham, Buckinghamshire, UK). After 1 min in the presence of Ca^{2+}, the nerves were washed three times (10 sec each) by sequential transfer to tubes containing K-B in which $CaCl_2$ was substituted by $LaCl_3$ (10 mM). The nerve segment proximal to the ligature was then cut into two pieces (1 cm long each). Each 1-cm segment was transferred to a tube containing 500 μl of a tissue solubilizer (Solvable; New England Nuclear, Boston, MA). After warming (3 hr at 60°), 400 μl of the mixture containing the solubilized tissue were transferred to a vial containing 20 ml of scintillation liquid (Formula 989; New England Nuclear) and counted in a Beckman LS2800 scintillation counter.

Chemicals. Ouabain and disodium ATP were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase was from Boehringer-Mannheim. [^{3}H]Ouabain and [^{3}H]NA were purchased from New England Nuclear. $^{45}CaCl_2$ was purchased from Amersham. All other reagents were obtained from commercial sources and were of the highest quality available.

Results

Axoplasmic transport of [^{3}H]ouabain binding sites. [^{3}H]Ouabain was specifically bound to a crude homogenate membrane preparation from nonligated cat hypogastric nerves. Scatchard analysis of the data showed a single, high affinity, binding site, with a K_d of 10.85 nM and a B_{max} of 260 fmol/mg of protein (Fig. 1).

[^{3}H]Ouabain binding sites were transported along the hypogastric nerve. Consequently, the number of [^{3}H]ouabain binding sites in the proximal part of the ligature increased with the time of ligation, reaching a maximum 48 hr after ligation (about 1 pmol/mg of protein) (Fig. 2). No significant changes in K_d values with time of ligation were observed. As expected, after

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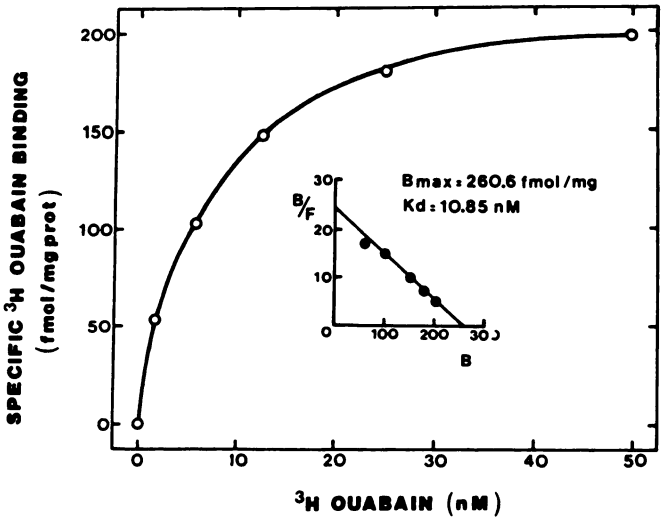

Fig. 1. Saturation isotherm for [³H]ouabain (1–50 nM) binding to homogenates from nonligated cat hypogastric nerves. Unlabeled ouabain (100 μM) was used to determine nonspecific binding. *Inset*, Scatchard analysis revealed a K_d of 10.85 nM and a B_{max} of 260.6 fmol/mg of protein. For further details, see Materials and Methods.

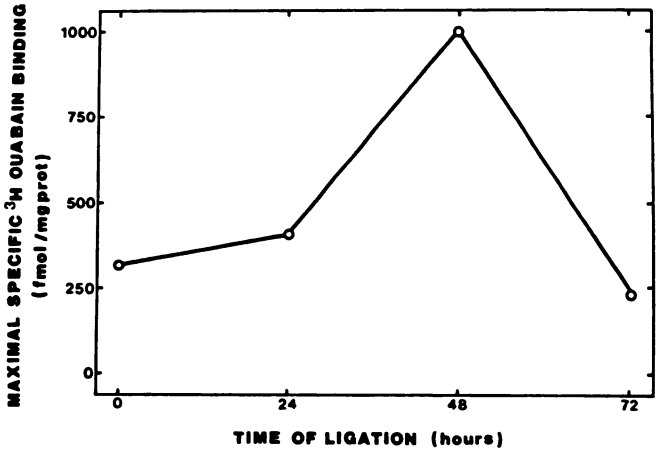

Fig. 2. Axoplasmic transport of [³H]ouabain binding sites in cat hypogastric nerves. The maximal [³H]ouabain binding capacity in membranes from hypogastric nerves is plotted as a function of the time of ligation. Each *point* represents a single experiment done with membranes obtained by pooling nerves from six cats ligated at the times showed.

48 hr of ligation the number of binding sites decreased to basal levels (around 250 fmol/mg of protein) (Fig. 2), suggesting nerve degeneration, as previously described for other ligands in this preparation (16). Specific [³H]ouabain binding to 1-cm nerve segments, from 48-hr ligated cat hypogastric nerves, that had been taken not in the vicinity of the ligature but 1–2 cm from the ligature showed B_{max} values similar to those obtained in nonligated hypogastric nerves (data not shown). This result is in good agreement with previous data showing that only the nerve segment in close proximity (1 cm) to the ligature achieves the properties of a sympathetic nerve terminal (8, 12–16).

Dose-response curve for ouabain-induced [³H]NA secretion. After the internal CA stores of the ligated nerve were labeled with [³H]NA and the labeled compound that had not been taken up was washed off, the nerve trunk was transferred to a Ca^{2+}-free medium and incubated for 60 min in the presence of ouabain, as described in Materials and Methods. CA secretion was evoked by Ca^{2+} reintroduction in the presence of ouabain. Fig. 3 shows the time course of [³H]NA secretion induced by ouabain (1 mM) from a 48-hr ligated cat hypogastric nerve.

According to our hypothesis, ouabain induced a dose-dependent NA secretion from 48-hr ligated cat hypogastric nerves (Fig. 4). Calcium reintroduction, without pretreatment with ouabain, did not evoke a significant NA efflux (data not shown), suggesting that prior blockade of Na^+,K^+-ATPase is required to induce CA secretion upon Ca^{2+} reintroduction. The secretory effect of ouabain became apparent at doses above 1 μM and reached a maximum at 1 mM (ED_{50} around 50 μM) (Fig. 4).

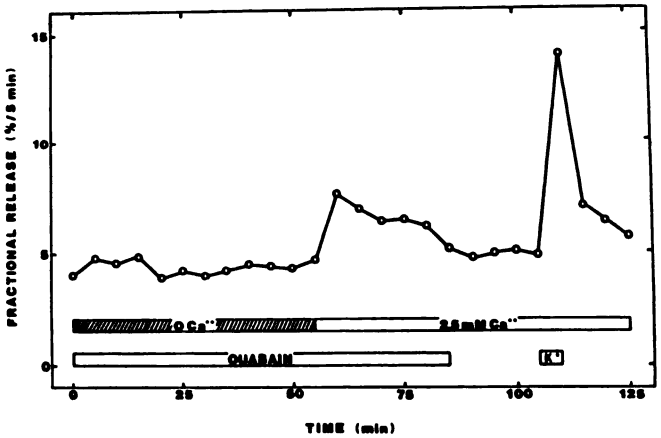

Fig. 3. Time course of [³H]NA secretion, induced by ouabain (1 mM), from a hypogastric nerve ligated 48 hr earlier. For further details, see Materials and Methods.

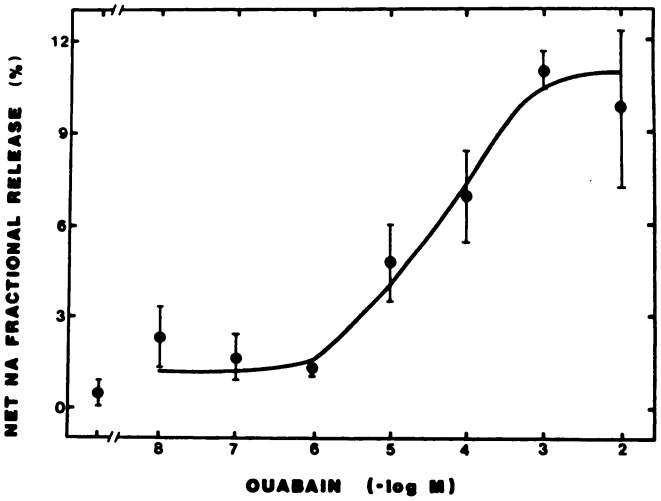

Fig. 4. Dose-response curve for ouabain-induced [³H]NA secretion. [³H]NA secretion from 48-hr ligated cat hypogastric nerves was elicited by Ca^{2+} reintroduction in nerves treated with ouabain, at the doses showed, in the absence of Ca^{2+}. Secretions obtained at ouabain doses above 10 μM are significantly different from those obtained at ouabain doses smaller than 1 μM ($p < 0.05$) or from secretion produced by Ca^{2+} reintroduction in the absence of ouabain ($p < 0.001$). Data represent mean ± standard error of four to six experiments.

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Sodium dependency of ouabain-induced [^{3}H]NA secretion. It is generally accepted that CA secretion in response to ouabain is mediated by activation, in reverse mode, of a Na^+/Ca^{2+} exchange mechanism (7–10). Accordingly, changes in $[Na^+]_o$ would affect the amount of CA secretion in response to a fixed amount of ouabain (9). In the ligated cat hypogastric nerve, ouabain-induced [^{3}H]NA secretion was dependent on $[Na^+]_o$, as has been shown for other adrenergic tissues (9). When $[Na^+]_o$ was reduced to 25 mM, the net fractional release evoked by ouabain (1 mM) during a 30-min period amounted to 3% of the total [^{3}H]NA content in the cell. However, when Na^+ was raised in the extracellular medium, secretion increased markedly, reaching a plateau at a Na^+ concentration of 100 mM (net fractional release, ~9%) (Fig. 5). Taken together, these data suggest the presence, in the ligated cat hypogastric nerve, of a functional Na^+/Ca^{2+} exchange mechanism.

Ouabain-induced $^{45}Ca^{2+}$ uptake. After ligation of the hypogastric nerve, the segment proximal to the ligature develops several changes that convert it into a sympathetic nerve terminal. One of these changes is an increase in the ability to regulate $[Ca^{2+}]_i$. One way of achieving this could be to increase the number of Na^+/Ca^{2+} exchange molecules above the level observed in the nonligated axon. To study this possibility, we measured the $^{45}Ca^{2+}$ influx evoked by Ca^{2+} reintroduction after ouabain treatment. If the total amount of Ca^{2+} taken up by the 1-cm segment proximal to the ligature increased with the time of ligation, above levels in either nonligated nerves or segments of 48-hr ligated nerves taken between 1 and 2 cm proximal to the ligature, it would indicate that more, functionally active, Na^+/Ca^{2+} exchange carriers were being incorporated into the axolemma. A larger number of molecules able to extrude Ca^{2+} would be expected from nerve terminals over nerve trunks.

As can be observed in Fig. 6, there was a substantial increase in Ca^{2+} uptake after Ca^{2+} reintroduction in 48-hr ligated cat hypogastric nerves, which amounted to 0.6 nmol/min/cm. The total amount of Ca^{2+} taken up by nerve segments 1–2 cm from the ligature was similar to that observed in nonligated nerves and amounted to around 0.1 nmol/min/cm.

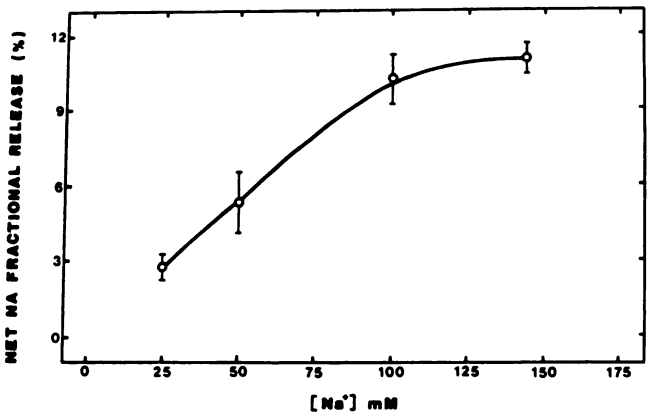

Fig. 5. Effect of $[Na^+]_o$ changes on ouabain-induced (1 mM) [^{3}H]NA secretion from 48-hr ligated cat hypogastric nerves. [^{3}H]NA secretion obtained at $[Na^+]_o$ of either 100 or 144 mM is significantly different from release obtained at $[Na^+]_o$ of 50 ($p < 0.05$) or 25 mM ($p < 0.001$). Data represent mean ± standard error of four to six experiments.

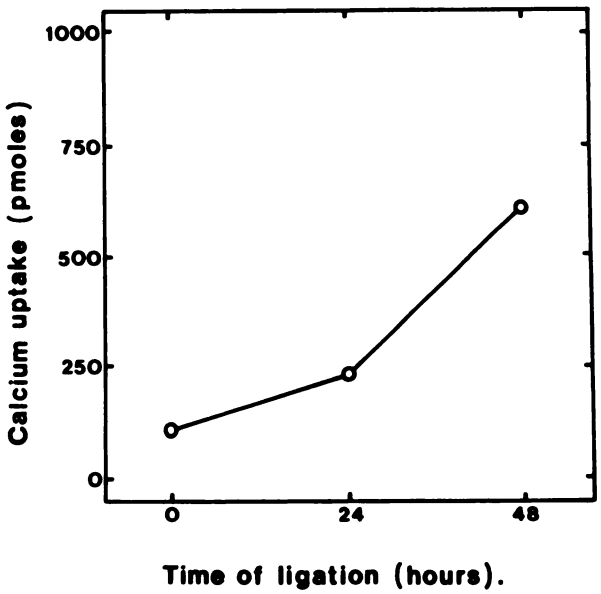

Fig. 6. Calcium uptake (pmol/min/cm) by ligated cat hypogastric nerves is plotted as a function of the time of ligation. Calcium uptake, after ouabain treatment, was induced as described in Results. Each *point* represents the average of two different experiments. Individual values for each determination are within the size of the symbol.

Discussion

We have shown here that the ligated cat hypogastric nerve contains binding sites for [^{3}H]ouabain that are transported down the axon and accumulate proximally to a ligature. Blockade of the enzyme Na^+,K^+-ATPase, accumulated in the nerve trunk, by increasing doses of ouabain caused a graded NA secretion that was dependent on the presence of both Ca^{2+} and Na^+ in the extracellular medium. In addition, Ca^{2+} uptake evoked by Ca^{2+} reintroduction after ouabain treatment increased with time of ligation. Taken together, these results strongly support the idea that the ligated cat hypogastric nerve is equipped with a functional Na^+/Ca^{2+} exchange mechanism that could contribute to regulation of $[Ca^{2+}]_i$ in this preparation. Additionally, the number of Na^+/Ca^{2+} exchange carrier molecules increased with time of ligation.

It is generally accepted that CA secretion from sympathetic tissues in response to ouabain is dependent upon the presence of Na^+ in the extracellular medium (9). The proposed mechanism of action for the cardiac glycoside-induced CA release is the following. Blockade of Na^+,K^+-ATPase located in the plasma membrane (20) would increase $[Na^+]_i$ (21). This would lead to activation, in reverse mode, of the Na^+/Ca^{2+} exchange mechanism (22), causing an increase in $[Ca^{2+}]_i$ (22, 23) and, accordingly, CA secretion. Following this hypothesis, it would be expected that ouabain-induced NA secretion from the ligated cat hypogastric nerve would be proportional to $[Na^+]_o$ and dependent on the presence of Ca^{2+} in the extracellular medium. Our data indicate that this is so and that the mechanism of ouabain-induced NA secretion in the ligated hypogastric nerve is similar to that present in the adrenal medulla (11) and in sympathetic nerve terminals (9). This fact strongly supports the idea that the ligated cat hypogastric nerve has a functionally

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active Na^+/Ca^{2+} exchange mechanism similar to that present in other adrenergic tissues.

The total amount of Ca^{2+} taken up by 1-cm segments proximal to the ligation increases substantially with the time of ligation, compared with either nonligated nerves or segments taken between 1 and 2 cm proximal to the ligature in 48-hr ligated nerves. These result suggest that, by stopping axoplasmic flow, there is not only accumulation of transported molecules but also incorporation of those molecules, physiologically bound for the nerve terminals, into the axolemma. The result of this process is the transformation of a nerve trunk into a sympathetice nerve terminal free of effector cells.

The affinity of [^{3}H]ouabain for its binding site found in this study (~10 nM) agrees very well with the affinity reported for the high affinity binding site described in other neural tissues, such as rat brain (24) and rat pineal (19). However, there is an important discrepancy between the affinity constant for [^{3}H] ouabain obtained by binding studies (~10 nM) and the dose of ouabain required to induce CA secretion from the ligated cat hypogastric nerve (>1 μM) (Fig. 4). One possible explanation is that, in the same way as in other neural tissues (19), the ligated cat hypogastric nerve might contain two different forms of Na^+,K^+-ATPase, one with high affinity and another with low affinity for ouabain. Our inability to obtain evidence of the presence of a low affinity [^{3}H]ouabain binding site could be due to technical limitations, because this binding site appears to have a very rapid dissociation rate (25).

The axonal transport of [^{3}H]ouabain binding sites in the hypogastric nerve is similar to that described in the rat sciatic nerve (26). The accumulation of [^{3}H]ouabain binding sites follows the same temporal pattern observed for other molecules in the ligated cat hypogastric nerve, i.e., dopamine β-monooxygenase, noradrenaline, and cholinoceptors (12); there is a peak of accumulation at 48 hr, and after that time the accumulation declines, probably due to nerve degeneration (16). In addition, it is important to note that at least part of the accumulated Na^+,K^+-ATPase must be externalized in the axolemma, because its blockade by ouabain, a highly polar drug, induces CA secretion from this ligated adrenergic nerve trunk.

It is possible that, similarly to the incorporation of Na^+/Ca^{2+} exchange carrier molecules after ligation, some incorporation of [^{3}H]ouabain binding sites into the axolemma takes place. This possibility cannot explain the accumulation of [^{3}H]ouabain binding sites in the 1-cm segment proximal to the ligature, because the level of [^{3}H]ouabain specific binding observed in segments from 48-hr ligated nerves, taken between 1 and 2 cm proximal to the ligature, is similar to that observed in nonligated nerves. However, due to the filtration technique used in the assay, it is possible that we might miss some of the soluble [^{3}H]ouabain binding sites. This would imply not the absence of axoplasmic transport but an underestimation of it. Another possibility to be excluded is local synthesis, proximal to the ligature, of [^{3}H]ouabain binding sites. However, electromicroscopic studies did not show the presence of ribosomal structures proximal to the ligature.[1]

In conclusion, our data present evidence that the ligated cat hypogastric nerve has a functionally active Na^+/Ca^{2+} exchange system that could contribute to maintaining $[Ca^{2+}]_i$ at resting levels. The results presented in this paper, together with previously published work (8, 12–16), fully validate, from a biochemical point of view, the ligated cat hypogastric nerve as a model of giant adrenergic nerve terminal free of effector cells. This will allow the study of different biological problems of sympathetic neurons without the interference of effector cells. Further work is in progress to study the immunocytochemistry of this giant sympathetic nerve terminal.

[1] V. Ceña, S. Calvo, C. González-García, I. Fariñas, and J. Marsal, unpublished observations.

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