Ouabain-evoked Norepinephrine Release from Intact Rat Sympathetic Neurons: Evidence for Carrier-mediated Release¹

KATHLEEN J. SWEADNER

Neurosurgical Research, Massachusetts General Hospital, Boston, Massachusetts 02114 and Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

Abstract

Inhibition of the Na,K-ATPase is known to cause transmitter release from many neurons. The mechanism of [³H]norepinephrine release was examined in primary cultures of sympathetic neurons. Ouabain caused [³H]norepinephrine release at concentrations that produced 80 to 90% inhibition of Na,K-ATPase activity. The effect of ouabain was compared with the effects of high K⁺ and the Ca²⁺ ionophore A23187. [³H]Norepinephrine release elicited by depolarization with high extracellular K⁺ was dependent on extracellular Ca²⁺, was unaffected by tetrodotoxin, was potentiated by reducing extracellular Na⁺, and was potentiated by the norepinephrine uptake inhibitor desipramine. These are the results expected if high K⁺ causes release by exocytosis, and if blockade of the Na⁺-dependent norepinephrine uptake system increases the net measurable release of the transmitter. The Ca²⁺ ionophore A23187 caused [³H]norepinephrine release that was not dependent on extracellular Ca²⁺ but which was like the release elicited by high K⁺ in other respects. Release elicited by ouabain was independent of extracellular Ca²⁺, was delayed for several hours by tetrodotoxin, was inhibited by reducing the concentration of extracellular Na⁺, and was inhibited by desipramine. These results suggest that the measured increase in transmitter release is secondary to a rise in the concentration of intracellular Na⁺. The data are consistent with the hypothesis that at high levels of pump inhibition, ouabain elicits nonvesicular [³H]norepinephrine release through reversal of the Na⁺-dependent plasma membrane carrier.

Birks (1963) first investigated the effect of inhibition of the Na⁺ pump on neurotransmitter release, after pointing out that the changes in intracellular ion concentrations produced are similar to those that would be expected to accompany repetitive nerve activity. Since then, a number of investigators have confirmed that cardiac glycosides elicit transmitter release in the autonomic nervous system, in the brain, at the neuromuscular junction, and in the adrenal medulla (see Powis, 1983, for review). The mechanism remains controversial, however. Inhibition of the Na,K-ATPase could lead to depolarization

of the neuron, followed by Ca²⁺ influx and transmitter release by exocytosis (Banks, 1967). It could also lead indirectly to a rise in intracellular Ca²⁺, through mobilization of intracellular Ca²⁺ stores (Baker and Crawford, 1975), through Ca²⁺ exchange for intracellular Na⁺ (Nakazato et al., 1978), or through inhibition of Ca²⁺ efflux (Pocock, 1983a, b), which again would permit transmitter release by exocytosis. Raiteri and Levi (1978) have proposed that an increase in intracellular Na⁺ permits the nonvesicular efflux of transmitters by reversal of Na⁺-dependent plasma membrane carriers. Vizi et al. (1982) argue that inhibition of the Na,K-ATPase somehow directly results in transmitter release through unidentified membrane pathways, independent of changes in intracellular ion concentrations.

The fact that evidence has been presented in support of all of these models suggests that there is considerable heterogeneity in experimental preparations, such as in the properties of membrane pumps and channels. There are two molecular forms of the Na,K-ATPase, for example, which have different biochemical properties and different affinities for the cardiac glycosides (Sweadner, 1979). The two forms can be expressed in different kinds of cells (Specht and Sweadner, 1984), and it is not known which form is expressed in preparations used previously to investigate the role of the Na,K-ATPase in transmitter release. In addition, the excision, slicing, or homogenization of a tissue may affect membrane leakiness, which could affect the steady-state metabolism of ions. It would be advantageous to study cells which have not been mechanically disturbed and in which the Na,K-ATPase has been identified.

In the experiments described here, the controversy has been addressed by examining the mechanism of ouabain-evoked release of [³H]norepinephrine from primary cultures of sympathetic neurons, which express the form of the Na,K-ATPase with a low affinity for cardiac glycosides (Sweadner, 1979). Sympathetic neurons from neonatal rats can be maintained in primary cell culture in the virtual absence of other cell types, where they express the physiological, biochemical, and morphological characteristics of such neurons in the adult (Landis, 1976; O'Lague et al., 1978; Patterson, 1978). The cultures release endogenous norepinephrine when stimulated electrically (Furshpan et al., 1976). They also accumulate and store exogenous [³H]norepinephrine in a reserpine-sensitive pool and release it when treated with agents that are thought to elicit the exocytosis of synaptic vesicles, such as elevated extracellular K⁺ (Patterson et al., 1976) and black widow spider venom (Sweadner, 1983). Such cultures are favorable for the study of the ionic basis of neurotransmitter release because the cells are intact, and because the uptake and efflux of ions from other cell types is eliminated.

Materials and Methods

Cell cultures. Primary cultures of postmitotic sympathetic neurons from the superior cervical ganglion of the newborn CD rat (Charles River Breeding Laboratories, Wilmington, MA) were prepared and maintained as described previously by Hawrot and Patterson (1979), in medium containing 5.4 mM K⁺ and no conditioned medium. Cells were plated in 8-mm-diameter wells formed

Received October 11, 1984; Revised February 13, 1985;
Accepted February 15, 1985

¹ This work was supported by National Institutes of Health Grant NS 18233. K. J. S. is an Established Investigator of the American Heart Association and a Research Fellow of the Alfred P. Sloan Foundation. Ms. Heather Shutt gave invaluable assistance in the maintenance of cultures and in the execution of experiments. Desipramine was generously provided by Dr. W. J. Hudak, Merrell Dow Laboratories, Cincinnati, Ohio.

by boring holes in 35-mm Petri dishes and sealing plastic coverslips to the holes with wax. Cells (500 to 1000/well) were plated on a substratum of dried collagen, and the cultures were maintained in bicarbonate-containing medium in the presence of nerve growth factor (the gift of A. Doupe, Harvard Medical School) for 3 to 6 weeks before use. Non-neuronal cells were eliminated during the first week after plating by treatment with arabinosylcytosine. All solutions used on living cultures were prepared in deionized and glass-distilled water, using only disposable, sterile tissue culture flasks and pipettes to avoid contamination with detergents and other chemicals.

[³H]Norepinephrine uptake and release. Norepinephrine release was assessed by measuring the release of tritium from cultures which had accumulated exogenous [³H]norepinephrine. Cells were allowed to accumulate L-[2,5,6-³H]norepinephrine or DL-[7-³H]norepinephrine (New England Nuclear, Boston, MA) during a 1-hr incubation with 10⁻⁶ M exogenous [³H]norepinephrine (specific activity, 10.8 to 54.8 Ci/mmol) in 1 ml of culture medium. Cultures of sympathetic neurons containing 500 to 1000 cells typically accumulated 1 to 2% of the [³H]norepinephrine available to them during a 1-hr preincubation. The cultures were then washed three times with minimal salts solution (140 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl₂, 1.2 mM CaCl₂, 10 mM glucose, and 1.5 mM HEPES/Tris buffer, pH 7.0) and were preincubated in 50 μ l of minimal salts solution for 30 min to 1 hr to allow the deamination and excretion of cytoplasmic [³H]norepinephrine. Patterson et al. (1976) found that under these conditions the majority of [³H]norepinephrine remaining in the cells is reserpine sensitive and is stored in a particulate fraction, presumably the synaptic vesicles. As reported previously (Burton and Bunge, 1975; Patterson et al., 1976), uptake of exogenous [³H]norepinephrine was blocked by 10⁻⁵ M desipramine, and the stored label was released upon treating the cells with 2 $\times$ 10⁻⁶ M reserpine or 10⁻³ M tyramine (data not shown). This is consistent with the established evidence that there is a desipramine-sensitive plasma membrane carrier for norepinephrine (Iversen, 1975), as well as reserpine-sensitive, tyramine-displaceable storage of the norepinephrine in a synaptic vesicle pool.

After the neurons were loaded with exogenous [³H]norepinephrine, the release of tritium was followed by changing the minimal salts solution every hour and measuring the radioactivity released and that remaining in the cells at the end of the experiment. Cultures were dissolved in 1% sodium dodecyl sulfate, and radioactivity was counted by liquid scintillation in Ultrafluor (National Diagnostics, Somerville, NJ). The data are displayed as the log of the total radioactivity remaining in the cells versus time; when release occurs as a simple exponential, this permits expression of the rate of release as a half-time and corrects for the depletion of labeled intracellular stores. The absolute rates of release, both basal and stimulated, varied between platings but were very reproducible when cultures from the same plating were compared. The experiments shown are representative results obtained with cultures from 14 different platings, and all experiments were repeated with cultures from at least two platings.

Release experiments were performed in minimal salts solution instead of complete medium to avoid the fluctuations of pH attendant on using bicarbonate-containing media. Control experiments performed in complete culture medium, including nerve growth factor, serum, and bicarbonate, gave the same results as experiments performed in minimal salts solution with glucose.

Assay of Na,K-ATPase inhibition. Because the inhibition of the Na,K-ATPase by cardiac glycosides is slow, a continuous spectrophotometric assay was used to monitor the rate of hydrolysis of ATP so that equilibrium inhibition values could be measured. ATP hydrolysis was coupled to the oxidation of nicotinamide adenine dinucleotide (NADH) using pyruvate kinase and phosphoenolpyruvate to regenerate ATP and produce pyruvate, and lactate dehydrogenase to reduce pyruvate and oxidize NADH (Pullman et al., 1960). The reaction was monitored at 340 nm in a Perkin-Elmer Lambda 3 spectrophotometer equipped with a thermoelectric cell holder set for 37°C. Hydrolysis in the absence of cardiac glycoside was linear with time provided that vanadium-free ATP was used (grade I ATP from Sigma Chemical Co., St. Louis, MO). ATP hydrolysis in the presence of cardiac glycoside declined slowly with time but reached a steady state within, at most, 20 to 30 min. Because of the limited quantities of sympathetic neurons available, membranes prepared from cultured rat brain non-neuronal cells (Sweadner, 1979) were substituted in these experiments. Previous work indicated that cultured sympathetic neurons and glia express the same form of the Na,K-ATPase by the criteria of cardiac glycoside affinity and subunit electrophoretic mobility (Sweadner, 1979), and more recent evidence has been obtained that they are antigenically cross-reactive as well (K. J. Sweadner and R. C. Gilkeson, manuscript in preparation). The concentrations of NaCl and KCl were those used in the minimal salts solution. CaCl₂ was omitted, and choline chloride was added as appropriate. ATP and MgCl₂ were both 3 mM, and the buffer was 30 mM HEPES/Tris, pH 7.2.

Materials. The Ca²⁺ ionophore A23187 was obtained from Calbiochem-Behring (La Jolla, CA). A stock solution prepared in dimethyl formamide was found to lose activity even when protected from light and stored at -20°C; therefore, a fresh solution was prepared for each experiment. Desipramine was obtained from Merrell Dow Pharmaceuticals Inc. (Cincinnati, OH). Ouabain, strophanthidin, veratridine, tetrodotoxin, pargyline, reserpine, ATP, phosphoenolpyruvate, NADH, lactate dehydrogenase, and pyruvate kinase were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Release elicited by inhibition of the Na,K-ATPase. The concentration of ouabain required to elicit [³H]norepinephrine release from sympathetic neurons was compared with that required to inhibit the Na,K-ATPase *in vitro* to estimate what fraction of pump activity must be inactivated for a measurable effect (Fig. 1). The rate of release is expressed as the ratio of the half-time in untreated cells to the half-time in treated cells, to correct for plating-to-plating variations (up to 2-fold) in the basal rate of release. The activity of the enzyme was assayed *in vitro* in a reaction mixture that simulated the ionic conditions in the extracellular medium except for the presence of 3 mM ATP and 3 mM MgCl₂, and the absence of 1.2 mM CaCl₂. Na⁺ and K⁺ concentrations typical of the extracellular environment were used instead of intracellular concentrations because the affinity of the ouabain-binding site, which faces the extracellular medium, is known to be affected by extracellular K⁺ (Schwartz et al., 1975). Ouabain inhibited the Na,K-ATPase with a K_{0.5} of 3 $\times$ 10⁻⁵ M, whereas ouabain- or strophanthidin-elicited norepinephrine release had a much steeper concentration dependence. Accelerated release was seen only after a threshold was exceeded, when 80% or more of the Na,K-ATPase was inhibited.

Ouabain had no effect on norepinephrine release at concentrations less than 10⁻³ M, but at exactly 10⁻³ M it was observed in different replicate experiments to have three different kinds of effects: (1) no effect, (2) a rate of release intermediate between the control and that seen at 3 $\times$ 10⁻³ M ouabain, and (3) the effect illustrated in Figure 2, where release occurred at the control rate for

Figure 1. Concentration dependence for the inhibition of the Na,K-ATPase by ouabain, and for the stimulation of norepinephrine release by ouabain or its congener, strophanthidin. Inhibition of the Na,K-ATPase was measured in a membrane preparation obtained from cultured rat brain glia, which express the same form of the Na,K-ATPase that is expressed by cultured rat sympathetic neurons. Ouabain-insensitive ATPase activity, which was approximately 50% of the total ATPase activity, was subtracted from the data shown. ■, ouabain-sensitive ATPase activity. The relative rate of norepinephrine release is expressed as the ratio of the half-time of release in the presence of ouabain (○) or strophanthidin (●) to the half-time of release in its absence. Each point is the result of a separate experiment such as those illustrated in the other figures. The majority of the radioactivity released in control cultures is actually in the form of metabolites of norepinephrine (data not shown). Therefore, the relative rates of release in the presence and absence of ouabain or strophanthidin are likely to be minimum estimates.

Figure 2. Threshold effect of ouabain on the release of [3 H]norepinephrine. ●, control; ○, 3×10^{-4} M ouabain; ■, 10^{-3} M ouabain; □, 3×10^{-3} M ouabain.

2 hr and then abruptly increased to the rate seen in the presence of 3×10^{-3} M ouabain. Such a delayed effect of ouabain suggests that release is a result of cumulative changes inside the cells, rather than of an immediate consequence of inhibition of the pump.

It is possible that ouabain might elicit transmitter release at lower concentrations if the cells were electrically active and, thus, had increased fluxes of either Na^+ or Ca^{2+} . Accordingly, the effect of ouabain was examined in the presence of 54 mM K^+ or of low levels (10^{-5} M) of A23187. As in unstimulated cells, however, no consistent effect of ouabain could be demonstrated at concentrations of 10^{-3} M or lower (data not shown).

The high concentrations of ouabain required to elicit release suggest that the release may be in response to toxic conditions. Evidence that prolonged ouabain treatment is toxic comes from the observation that in some experiments it resulted in unmistakable deterioration of neurite structure (Fig. 3). The neuronal cell bodies in the cultures still looked similar to those in the controls, both by Nomarski and by phase contrast optics (not shown). When the cultures were fed complete culture medium and returned to the incubator, the cell bodies survived for more than a week, but there was little detectable recovery of normal axonal structure. Similar observations were made by Birks (1962) with electron microscopy of ouabain-treated superior cervical ganglia and neuromuscular junctions. However, with the cultured sympathetic neurons used here, the light microscopic appearance of the neuronal processes was always normal for the first several hours of ouabain treatment, and in fact structural deterioration was not seen consistently even after 6 hr in ouabain. There appear to be plating-to-plating differences in the ability of the cells to withstand the insult.

Norepinephrine release in response to conventional stimuli. To establish a basis for comparison with ouabain, elevated extracellular K^+ and the Ca^{2+} ionophore A23187 were tested for their ability to elicit the release of [3 H]norepinephrine (Fig. 4, A and B). Elevated extracellular K^+ depolarizes the cells and is thought to permit the entry of Ca^{2+} through voltage-sensitive channels (Blaustein et al.,

1972). K^+ at 54 mM reduced the half-time of [3 H]norepinephrine release from >360 min to 66 min in the presence of extracellular Ca^{2+} , but had very little effect when Ca^{2+} was omitted from the solutions (Fig. 4A). A23187 is a Ca^{2+} ionophore that has been observed to induce exocytotic release in a variety of cells (Pressman, 1976). A single application of A23187 at 3×10^{-5} M was sufficient to sustain a rapid rate of norepinephrine release for several hours (Fig. 4B); the drug readily penetrates the membrane and does not appear to wash out of the cells. The ionophore caused release even in the nominal absence of extracellular Ca^{2+} , suggesting that it may release Ca^{2+} from intracellular stores.

The dependence of norepinephrine release on exogenous Ca^{2+} was tested by omitting Ca^{2+} from the medium and replacing it with additional Mg^{2+} . In intact tissues, Ca^{2+} may leak from considerable intracellular stores into a restricted extracellular space, making it essential to use a Ca^{2+} buffer to keep the extracellular concentration low, but in the dilute environment of the cultures the omission of Ca^{2+} alone was sufficiently harsh to cause the cells to deteriorate after a period of hours. Spontaneous release of [3 H]norepinephrine was unaltered for 4 to 6 hr (Fig. 4A; no Ca^{2+} control), but after prolonged Ca^{2+} deprivation, the cells released [3 H]norepinephrine at an accelerated rate by an unknown mechanism. This may have been a consequence of cell death; neurons incubated without Ca^{2+} for 24 hr deteriorated to the extent that they lost the ability to incorporate labeled L-leucine into protein (data not shown).

In the experimental protocol used, the cultures are in contact with the newly released [3 H]norepinephrine and are able to recapture some of it by high affinity uptake. To test the effect of recapture on the apparent rate of release, the experiments were repeated in the presence of the uptake inhibitor, desipramine (Iversen, 1975). Figure 5, A and B, shows that 10^{-5} M desipramine potentiates the apparent rate of release of [3 H]norepinephrine elicited both by high K^+ and by A23187. Desipramine had no detectable effect on the release of radioactivity from unstimulated cultures (not shown); such release has previously been shown to consist primarily of deaminated metabolites of norepinephrine, which are thought to diffuse passively from the cells (Patterson et al., 1976).

The desipramine-sensitive norepinephrine uptake from the extracellular medium is known to be due to an Na^+ -dependent carrier, and it should be possible to block it by reducing the concentration of extracellular Na^+ . The effect of reducing extracellular Na^+ to 14 mM, using choline as a substitute to maintain osmolality and ionic strength, was tested accordingly. Figure 6, A and B, shows that medium containing 14 mM Na^+ had little or no effect on the release

Figure 3. Light microscopic appearance of neuronal processes after extended treatment with ouabain. Neuronal processes were photographed at $\times 600$ with Nomarski optics. A, Control; B, after 6 hr in 10^{-5} M veratridine; C, after 6 hr in 10^{-3} M ouabain; D, after 6 hr in 3×10^{-3} M ouabain.

Figure 4. A, Norepinephrine release elicited by high K⁺ in the presence and absence of extracellular Ca²⁺. Normally the salts solution contains 140 mM Na⁺ and 5.4 mM K⁺. When 54 mM K⁺ was used, Na⁺ was reduced to 91.4 mM to maintain isotonicity. ●, control; ○, control minus Ca²⁺; ■, 54 mM K⁺; □, 54 mM K⁺ minus Ca²⁺. The ordinate shows the calculated percentage of the total [³H]norepinephrine remaining in the cells at each time point. B, Norepinephrine release elicited by the Ca²⁺ ionophore A23187 in the presence and absence of extracellular Ca²⁺. ●, control; ○, control minus Ca²⁺; □, A23187 (3 × 10⁻⁵ M); ■, A23187 minus Ca²⁺.

of radioactivity from control cultures, but it potentiated the release from cells stimulated with 54 mM K⁺ or A23187 much as did desipramine.

Ca²⁺ and Na⁺ dependence of the effect of ouabain. Ouabain and strophanthidin elicited rapid release of [³H]norepinephrine at concentrations of 3 × 10⁻³ M, and this release was not dependent upon the presence of Ca²⁺ in the extracellular medium (Fig. 7). As a control for the leakage of Ca²⁺ from intracellular pools into the extracellular medium, ouabain was also tested in a Ca²⁺-EGTA (ethyleneglycol bis-(β-aminoethyl ether)-N,N'-tetra-acetic acid) buffer with a final concentration of Ca²⁺ of 10⁻⁷ M (Graf and Penniston, 1981), with the same result.

If the effect of inhibition of the Na,K-ATPase on transmitter release were a consequence of a net rise in the concentration of intracellular Na⁺, any experimental condition that reduces Na⁺ influx might be predicted to block the effect of ouabain. Experiments were thus performed in a medium containing only 14 mM Na⁺ with choline as a substitute, instead of 140 mM Na⁺. Ouabain did not elicit the release of [³H]norepinephrine in 14 mM Na⁺ (Fig. 8). This contrasts with the potentiating effect of similar ionic conditions on release elicited by high K⁺ or A23187 (Fig. 6, A and B).

It is critical to know whether ouabain still inhibits the Na,K-ATPase in these altered ionic conditions; therefore, inhibition was measured *in vitro* in a reaction mixture containing 14 mM NaCl and 126 mM choline chloride and compared to the values obtained in the presence of 140 mM NaCl. As in the experiment shown in Figure 1, CaCl₂ was omitted from the standard minimal salts solution, and the reaction mixture contained 3 mM ATP and 3 mM MgCl₂. The result was that equilibrium half-maximal inhibition occurred at 3.1 × 10⁻⁵ M ouabain in 140 mM NaCl and at 3.3 × 10⁻⁵ M in 14 mM NaCl, 126

mM choline chloride. Since no transmitter release occurs with as much as 3 × 10⁻³ M ouabain in these ionic conditions, it would appear that inhibition of the Na,K-ATPase alone is not sufficient to cause the release.

Lowering extracellular Na⁺ would reduce the electrochemical driving force for Na⁺ entry and could have at least two consequences: it could reduce passive Na⁺ influx or it could reduce Na⁺:Ca²⁺ exchange. Tetrodotoxin at 10⁻⁵ M was found to delay the onset of ouabain-evoked [³H]norepinephrine release by 2 to 3 hr (Fig. 9). Although the voltage-sensitive Na⁺ gate is only one of several probable routes by which Na⁺ could enter the cell, it appears to make a significant contribution in the present experiments, supporting the hypothesis that net Na⁺ influx is the critical factor.

Norepinephrine release via a carrier. The results of Figures 8 and 9 are most simply interpreted as consistent with the hypothesis that ouabain-elicited norepinephrine release is the consequence of a rise in the concentration of intracellular Na⁺. A simple model to explain release of [³H]norepinephrine that is independent of extracellular Ca²⁺ and dependent on a rise in intracellular Na⁺ is that it could occur by efflux on the plasma membrane carrier instead of by exocytosis. To test this, desipramine was used to block the norepinephrine carrier while the Na,K-ATPase was inhibited with ouabain. Figure 10 shows that desipramine at 10⁻⁵ M completely blocked ouabain-elicited [³H]norepinephrine release. In other experiments it was determined that 10⁻⁶ M desipramine would block ouabain-elicited [³H]norepinephrine release by 30 to 40%, whereas 10⁻⁷ M desipramine was without effect (data not shown).

Norepinephrine release in veratridine. Veratridine depolarizes neurons by opening the voltage-sensitive Na⁺ channel, and it has been used by other investigators to elicit transmitter release (reviewed in

Figure 5. A, Norepinephrine release elicited by high K⁺ in the presence and absence of the norepinephrine uptake inhibitor desipramine. ●, control; ○, 54 mM K⁺; ■, 54 mM K⁺ plus 10⁻⁵ M desipramine. B, Norepinephrine release elicited by A23187 in the presence and absence of desipramine. ●, control; ○, A23187 (3 × 10⁻⁵ M); ■, A23187 plus 10⁻⁵ M desipramine.

Minchin, 1980). It caused release of [³H]norepinephrine from the cultured sympathetic neurons both in the presence and in the nominal absence of Ca²⁺ (Fig. 11A). The effect of veratridine was blocked by 10⁻⁵ M tetrodotoxin, as expected from its specificity for the Na⁺ channel, and, in fact, tetrodotoxin could restore the resting rate of [³H]norepinephrine release after 2 to 3 hr of veratridine-stimulated release (data not shown). In contrast to the release elicited by ouabain, however, the release elicited by veratridine was inhibited only approximately 50% by the carrier blocker desipramine (Fig. 11B).

Norepinephrine release in the absence of Na⁺. In Figure 6 it was shown that replacing all but 14 mM Na⁺ in the extracellular medium with choline had no effect on the resting rate of [³H]norepinephrine release. Complete removal of extracellular Na⁺ should have the effect of reversing the direction of the Na⁺ gradient across the membrane, and this would be predicted to drive the efflux of [³H]norepinephrine on the plasma membrane carrier even more effectively than the elevated intracellular Na⁺ concentrations produced by ouabain. As predicted, replacing all of the Na⁺ with choline elicited transmitter release (Fig. 12). Substitution of Li⁺ or *N*-methylglucamine for Na⁺ had a similar result, except that release rate tended to be higher. This effect of Na⁺ depletion did not depend on the presence of Ca²⁺ in the extracellular medium, and it was neither enhanced nor inhibited consistently by treating the cells with ouabain, tetrodotoxin, or desipramine (not shown). The problem with complete substitution of the Na⁺ in the extracellular medium was that the resulting transmitter release rates were usually highly variable, even when triplicate cultures from the same platings were compared. The complete removal of Na⁺ appears to have complex consequences that we do not understand; thus, no confidence can be placed on an analysis of its mechanism.

Discussion

The experiments reported here suggest that inhibition of the Na,K-ATPase with ouabain results in [³H]norepinephrine release from intact sympathetic neurons by a mechanism quite different from conventional release by exocytosis. The effect of ouabain was not abolished in the nominal absence of Ca²⁺. It required conditions permissive for passive Na⁺ influx, and it was inhibited by a drug, desipramine, which blocks the Na⁺-dependent norepinephrine carrier (Iversen, 1975; Raiteri and Levi, 1978). The simplest explanation is that ouabain permits intracellular Na⁺ levels to rise to the point that the carrier can run backwards: not necessarily at a rate comparable to its normal influx mode, but at a rate sufficient to allow a net efflux of the transmitter from the cell. Both high K⁺ and ouabain treatments are capable of producing nearly complete depletion of intracellular norepinephrine, which implies an equilibrium between the vesicular and cytoplasmic pools.

The mechanism of ouabain-elicited release. The most widely accepted model for transmitter release is that it occurs by exocytosis, dependent on an influx of extracellular Ca²⁺. Three laboratories have found ouabain-elicited catecholamine release from the adrenal medulla that is dependent on the presence of millimolar levels of exogenous Ca²⁺ (Banks, 1967; Garcia et al., 1980; Wakade, 1981), and a similar observation has been made for norepinephrine release from sympathetic neurons innervating the vas deferens (Nakazato et al., 1978). Other laboratories have found ouabain-evoked release that is only partially dependent on extracellular Ca²⁺: in vascular smooth muscle (Lorenz, Powis, Vanhoutte, and Shephard, 1980; Palaty, 1981) and in skeletal muscle (Elmqvist and Feldman, 1965; Birks and Cohen, 1968; Baker and Crawford, 1975; Branisteanu et al., 1979; Vizi and Vyskocil, 1979). A lack of dependence on extracellular Ca²⁺ is not in itself sufficient evidence to argue that

Figure 6. A, Norepinephrine release elicited by high K⁺ in the presence and absence of reduced extracellular Na⁺. ●, control; □, control with 14 mM Na⁺ and 126 mM choline chloride; ■, 54 mM K⁺ and 91.4 mM Na⁺; ○, 54 mM K⁺, 14 mM Na⁺, and 77.4 mM choline chloride. B, Norepinephrine release elicited by A23187 in the presence and absence of reduced extracellular Na⁺. ●, control; ○, control with 14 mM Na⁺ and 126 mM choline chloride; □, A23187 (10⁻⁵ M); ■, A23187 in 14 mM Na⁺ and 126 mM choline chloride.

transmitter release is nonvesicular, since vesicular release may result from the mobilization of intracellular Ca²⁺ stores or a reduction in Ca²⁺ efflux. Catecholamine release from the adrenal medulla has been shown to be accompanied by the release of the chromaffin granule protein dopamine β -hydroxylase both when release was Ca²⁺ dependent (Garcia et al., 1980) and when it was Ca²⁺ independent (Lastowicka and Trifaro, 1974; Pocock, 1983a). In these cases, it is very likely that release was occurring by exocytosis. Unfortunately, we have been unable to detect release of dopamine β -hydroxylase from rat sympathetic neurons under any experimental condition; thus, we have no positive evidence for the absence of vesicular release in the present experiments (data not shown).

The results presented in this report do not rule out an effect of ouabain on Ca²⁺ influx, Na⁺:Ca²⁺ exchange, or the mobilization of Ca²⁺ from intracellular stores, but they can be most simply explained by a minimal model which requires only that the concentration of intracellular Na⁺ rise and that transmitter leak from the cell as a result of the reversibility of an Na⁺-dependent membrane carrier. No dependence on extracellular Ca²⁺ could be demonstrated for the effect of ouabain, even when Ca²⁺ was buffered at 10⁻⁷ M, which eliminates the need to invoke depolarization-induced activation of Ca²⁺ channels or an increased Ca²⁺ influx through Na⁺:Ca²⁺ exchange. Another plausible model would be that Ca²⁺ efflux through Na⁺:Ca²⁺ exchange is decreased because of an increase in intracellular Na⁺, resulting in a higher intracellular level of Ca²⁺. This does not easily explain the effect of ouabain in causing release, however, because of the observed effect of reducing extracellular Na⁺. The presence of only 14 mM Na⁺ in the extracellular medium would be expected to have a qualitatively similar effect on Ca²⁺ efflux, and yet it blocks the effect of ouabain, rather than enhancing it. It is

simpler to suppose that reducing the concentration of extracellular Na⁺ limits the passive rise in intracellular Na⁺ which follows the inhibition of the Na⁺ pump. The effect of tetrodotoxin in delaying ouabain-elicited release strengthens this argument, since tetrodotoxin should act only to reduce Na⁺ influx. An increase in the concentration of intracellular Na⁺ may ultimately result in the release of Ca²⁺ from mitochondria (Carafoli, 1982), but there is no known reason to postulate an effect of Ca²⁺ on the desipramine-sensitive catecholamine carrier.

The hypothesis that transmitters can sometimes be released from neurons by the reversal of plasma membrane carriers is gaining experimental support. Specific uptake blockers have been shown to prevent release of norepinephrine or dopamine (Blażkowski and Bogdanski, 1972; Paton, 1973; Raiteri et al., 1977, 1979; Lorenz et al., 1980; Liang and Rutledge, 1982). Amphetamine-induced release of norepinephrine is also apparently carrier mediated (Rutledge, 1978). Amino acid transmitters may be released by an analogous mechanism (Levi and Raiteri, 1978; Schwartz, 1982; Yazulla and Kleinschmidt, 1983). Schwartz (1982) proposed that carrier-mediated GABA release was activated by depolarization, which is reasonable if there is a net movement of charge on the carrier. In other cases, high intracellular Na⁺ or reversal of the Na⁺ gradient was thought to permit the reversal of membrane carriers.

In slices and synaptosomes from brain, ouabain-evoked release of acetylcholine (Vizi, 1972; Meyer and Cooper, 1981; Vyas and Marchbanks, 1981) and serotonin (Collard and Williams, 1981) is seen that is independent of extracellular Ca²⁺. It has been proposed by Vizi (1972) that inhibition of the Na,K-ATPase is itself sufficient, independent of fluxes of either Na⁺ or Ca²⁺, to cause transmitter release by such means as destabilizing the membrane or changing

Figure 7. Norepinephrine release elicited by ouabain in the presence or absence of extracellular Ca^{2+} . ●, control; ○, control minus Ca^{2+} ; ■, ouabain (3×10^{-3} M); □, ouabain minus Ca^{2+} .

Figure 9. Norepinephrine release elicited by ouabain in the presence or absence of the voltage-sensitive Na^+ gate blocker, tetrodotoxin. ●, control; ■, ouabain (3×10^{-3} M); ○, ouabain plus tetrodotoxin (10^{-5} M).

Figure 8. Norepinephrine release elicited by ouabain in the presence or absence of reduced extracellular Na^+ . ●, control; ○, control with 14 mM Na^+ and 126 mM choline chloride; ■, ouabain (3×10^{-3} M); □, ouabain in 14 mM Na^+ and 126 mM choline chloride.

Figure 10. Norepinephrine release elicited by ouabain in the presence or absence of the norepinephrine carrier blocker, desipramine. ■, control; ●, ouabain (3×10^{-3} M); ○, ouabain plus desipramine (10^{-5} M).

Figure 11. A, Norepinephrine release elicited by the voltage-sensitive Na^+ gate toxin, veratridine, in the presence or absence of extracellular Ca^{2+} . ●, control; □, control minus Ca^{2+} ; ○, veratridine (3×10^{-5} M); ■, veratridine minus Ca^{2+} . B, Norepinephrine release elicited by veratridine in the presence or absence of desipramine. ●, control; ■, veratridine (10^{-6} M); ○, veratridine plus desipramine (10^{-6} M).

the intracellular pH. This model is clearly untenable here in view of the fact that the Na,K-ATPase can be inhibited by ouabain in the presence of reduced extracellular Na^+ , yet without any consequent release of transmitter. A dependence upon extracellular Na^+ (and by inference on Na^+ influx) has been demonstrated for the effect of ouabain on transmitter release in several laboratories (Birks, 1963; Birks and Cohen, 1968; Nakazato et al., 1978; Garcia et al., 1980; Pocock, 1983a). A reduction in extracellular Na^+ has also been observed to protect the neuron against the pathological changes in ultrastructure that are caused by prolonged poisoning with ouabain (Birks, 1962).

Some of the other apparent contradictions in the literature can be understood if one assumes that there are tissue- or cell-specific differences in the balance between exocytotic and carrier-mediated mechanisms. It is notable, for example, that no carrier-mediated release of catecholamines has been reported for the adrenal medulla, although that organ has been reported to have a high affinity uptake system for catecholamines analogous to the one in neurons (Kensberg and Trifaro, 1980; Role and Periman, 1983). Even Na^+ depletion is thought to cause exocytotic release in the adrenal medulla (Lastowecka and Trifaro, 1974; Nishimura et al., 1981). Veratridine, which opens the voltage-sensitive Na^+ gate, causes release from cultured sympathetic neurons that is independent of extracellular Ca^{2+} and that is at least partially inhibitable by desipramine. This contrasts with observations on rat brain synaptosomes, where the effect of veratridine was Ca^{2+} dependent and unaffected by desipramine (Mulder et al., 1975), and where it causes an increase in Ca^{2+} accumulation (Blaustein, 1975). Since veratridine depolarizes the cell, allowing Ca^{2+} to enter through voltage-sensitive channels as well as permitting diffusion-limited Na^+ entry through the Na^+

gate, it is plausible that either Ca^{2+} -dependent or Na^+ -dependent release mechanisms might predominate in different kinds of cells.

The lack of Na^+ in the extracellular medium would be predicted to cause release by reversing the direction of the Na^+ gradient, and yet desipramine had no consistent effect on norepinephrine release from sympathetic neurons in this condition. This may be due to a failure of the cells to maintain their integrity after prolonged Na^+ depletion. In synaptosomes, however, desipramine and nomifensine have been found to cause partial inhibition of norepinephrine and dopamine release into medium lacking Na^+ (Paton, 1973; Raiteri et al., 1977, 1979). Desipramine, like norepinephrine itself, may require extracellular Na^+ for binding to the carrier (Lee and Snyder, 1981), with the consequence that it is ineffective in inhibiting outward transport into medium lacking Na^+ at the concentrations used. Alternatively, desipramine may need to enter the cell by the Na^+ -dependent carrier before it can inhibit norepinephrine efflux.

Ouabain concentration and its physiological effects. The affinity of the Na,K-ATPase for ouabain can vary markedly depending on the species and on the concentration of K^+ (Schwartz et al., 1975) and, in certain species, on which of two different forms of the Na,K-ATPase catalytic subunit is present. The two forms of the Na,K-ATPase have been separated, and are named α , the kidney form, and $\alpha(+)$, the axolemma form, after their richest tissue sources (Sweadner, 1979). In the rat, the kidney form, α , has an affinity for ouabain of 3×10^{-5} M as reported here, whereas the axolemma form, $\alpha(+)$, has an affinity of 10^{-7} M (unpublished results).

It has been postulated that some of the physiological effects of ouabain on target tissues of the autonomic nervous system are a consequence of the release of neurotransmitter rather than of a direct effect of the drug on the target (reviewed by Gillis and Quest,

Figure 12. Norepinephrine release elicited by the lack of extracellular Na⁺ in the presence or absence of extracellular Ca²⁺. ●, control; □, control minus Ca²⁺; ■, 0 Na⁺, 140 mM choline chloride; ○, 0 Na⁺, 140 mM choline chloride minus Ca²⁺.

1980). A salient feature of the effect of ouabain on the heart is that it typically occurs at concentrations lower than those required to inhibit the cardiac Na,K-ATPase *in vitro*, a phenomenon that has resulted in much debate about the mechanism of ouabain inotropy. Lechat et al. (1983) have presented evidence that the inotropy seen at very low concentrations of ouabain in the guinea pig atrium may be due to the effects of autonomic transmitters. The low affinity form of the Na,K-ATPase is expressed in cultured sympathetic neurons of the rat (Sweadner, 1979); thus, it is not surprising that high concentrations of ouabain are required to elicit norepinephrine release here. However, the release of [³H]norepinephrine required a concentration of ouabain that is sufficient to cause nearly complete inhibition of even the low affinity Na,K-ATPase, which makes it unlikely that ouabain-elicited transmitter release can account for inotropy in the rat. The paradox could be explained if the sensitive cell, a sympathetic neuron or a cardiac cell, expressed the high affinity form of the catalytic subunit of the Na,K-ATPase *in vivo*. Alternatively, the inotropic effect could result not from increased norepinephrine release, but from reduced norepinephrine reuptake; it would be of interest to contrast the ouabain concentration dependencies for the inhibition of norepinephrine uptake and the stimulation of norepinephrine release.

The brain contains both forms of the Na,K-ATPase, whereas the forms expressed in most neurons innervating peripheral tissues is not known. It is possible that an apparent discrepancy in the sensitivities of different transmitters to release by ouabain is due to the use of a concentration of ouabain that is not sufficient to fully inhibit the low affinity form of the Na,K-ATPase. Raiteri et al. (1977), for example, found the release of norepinephrine from rat brain synaptosomes to be very insensitive to 10⁻⁴ M ouabain, whereas Raiteri et al. (1979) found the release of dopamine to be very sensitive in the same species and under similar experimental conditions. These investigators presented evidence that both transmitters leave the cell by plasma membrane carriers. Unless there are

fundamental differences in the properties of the norepinephrine and dopamine carriers, one would predict from these results that norepinephrine should be found in cells expressing the low affinity form of the Na,K-ATPase, and dopamine should be found in cells expressing the high affinity form. O'Fallon et al. (1981) found striking regional variations in the release of several transmitters from rat brain slices in response to 10⁻⁵ M ouabain, a concentration that would be expected to inhibit only the high affinity form of the Na,K-ATPase; the use of a concentration of ouabain high enough to inhibit the low affinity form as well might have given different results.

Summary. To evaluate the biological significance of ouabain-elicited transmitter release, we would like to know if it mimics any naturally occurring process, such as changes in intracellular ion concentrations following repetitive nerve activity or neuromodulator action. What remains to be determined is whether intracellular Na⁺ ever rises, even transiently, to levels comparable to those achieved after inhibition of the Na,K-ATPase. If they do, then reversal of the Na⁺-dependent carrier may be a normal mechanism of transmitter release. If they do not, then ouabain-elicited release may be of relevance only to cardiac glycoside toxicity and to ischemia.

References

- Baker, P. F., and A. C. Crawford (1975) A note of the mechanism by which inhibitors of the sodium pump accelerate spontaneous release of transmitter from motor nerve terminals. *J. Physiol. (Lond.)* 247: 209–226.
- Banks, P. (1967) The effect of ouabain on the secretion of catecholamines and on the intracellular concentration of potassium. *J. Physiol. (Lond.)* 193: 631–637.
- Birks, R. I. (1962) The effects of a cardiac glycoside on subcellular structures within nerve cells and their processes in sympathetic ganglia and skeletal muscle. *Can. J. Biochem. Physiol.* 40: 303–315.
- Birks, R. I. (1963) The role of sodium ions in the metabolism of acetylcholine. *Can. J. Biochem. Physiol.* 41: 2573–2597.
- Birks, R. I., and M. W. Cohen (1968) The action of sodium pump inhibitors on neuromuscular transmission. *Proc. R. Soc. Lond. (Biol.)* 170: 381–399.
- Blaszowski, T. P., and D. F. Bogdanski (1972) Evidence for sodium-dependent outward transport of the [³H]norepinephrine mobilized by calcium at the adrenergic synapse. Inhibition of transport by desipramine. *Life Sci.* 11: 867–876.
- Blaustein, M. P. (1975) Effects of potassium, veratridine, and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro*. *J. Physiol. (Lond.)* 247: 617–655.
- Blaustein, M. P., E. M. Johnson, Jr., and P. Needleman (1972) Calcium-dependent norepinephrine release from presynaptic nerve endings *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* 69: 2237–2240.
- Branisteau, D. D., B. Proca, and I. D. Haulicha (1979) Dual action of ouabain on transmitter release at neuromuscular junctions of the frog. *J. Pharmacol. Exp. Ther.* 209: 31–36.
- Burton, H., and R. P. Bunge (1975) A comparison of the uptake and release of [³H]norepinephrine in rat autonomic and sensory ganglia in tissue culture. *Brain Res.* 97: 157–162.
- Carafoli, E. (1982) The transport of calcium across the inner membrane of mitochondria. In *Membrane Transport of Calcium*, E. Carafoli, ed., pp. 109–139. Academic Press, Inc., New York.
- Collard, K. J., and S. R. Williams (1981) The effect of ouabain on the release of 5-hydroxytryptamine from superfused synaptosomes. *Biochem. Soc. Trans.* 9: 109–110.
- Elmqvist, D., and D. S. Feldman (1965) Effects of sodium pump inhibitors on spontaneous acetylcholine release at the neuromuscular junction. *J. Physiol. (Lond.)* 181: 498–505.
- Furshpan, E. J., P. R. MacLeish, P. H. O'Laigue, and D. D. Potter (1976) Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: Evidence for cholinergic, adrenergic, and dual-function neurons. *Proc. Natl. Acad. Sci. U. S. A.* 73: 4225–4229.
- Garcia, A. G., M. Hernandez, J. F. Horga, and P. Sanchez-Garcia (1980) On the release of catecholamines and dopamine-β-hydroxylase evoked by ouabain in the perfused cat adrenal gland. *Br. J. Pharmacol.* 68: 571–583.
- Gillis, R. A., and J. A. Quest (1980) The role of the nervous system in the cardiovascular effects of digitalis. *Pharmacol. Rev.* 31: 19–97.
- Graf, E., and J. T. Penniston (1981) CaATP: The substrate, at low ATP concentrations, of Ca²⁺ATPase from human erythrocyte membranes. *J. Biol. Chem.* 256: 1587–1592.

- Howrot, E., and P. H. Patterson (1979) Long-term culture of dissociated sympathetic neurons. *Methods Enzymol.* 58: 574-584.
- Iversen, L. L. (1975) Uptake processes for biogenic amines. In *Handbook of Psychopharmacology*, L. L. Iversen, S. D. Iversen, and S. H. Snyder, eds., Vol. 3, pp. 381-442, Plenum Press, New York.
- Kenigsberg, R. L., and J. M. Trifaro (1980) Presence of a high affinity uptake system for catecholamines in cultured bovine adrenal chromaffin cells. *Neuroscience* 5: 1547-1556.
- Landis, S. C. (1976) Rat sympathetic neurons and cardiac myocytes developing in microcultures: Correlation of the fine structure of endings with neurotransmitter function in single neurons. *Proc. Natl. Acad. Sci. U. S. A.* 73: 4220-4224.
- Lastowicka, A., and J. M. Trifaro (1974) The effect of sodium and calcium ions of the release of catecholamines from the adrenal medulla: Sodium deprivation induces release by exocytosis in the absence of extracellular calcium. *J. Physiol. (Lond.)* 236: 681-705.
- Lechat, P., C. R. Malloy, and T. W. Smith (1983) Active transport and inotropic state in guinea pig left atrium. *Circ. Res.* 52: 411-422.
- Lee, C.-M., and S. H. Snyder (1981) Norepinephrine neuronal uptake binding sites in rat brain membranes labeled with [3 H]desipramine. *Proc. Natl. Acad. Sci. U. S. A.* 78: 5250-5254.
- Levi, G., and M. Raiteri (1978) Modulation of γ -aminobutyric acid transport in nerve endings: Role of extracellular γ -aminobutyric acid and of cationic fluxes. *Proc. Natl. Acad. Sci. U. S. A.* 75: 2981-2985.
- Liang, N. Y., and C. O. Rutledge (1982) Evidence for carrier-mediated efflux of dopamine from corpus striatum. *Biochem. Pharmacol.* 31: 2479-2484.
- Lorenz, R. R., D. A. Powis, P. M. Vanhoutte, J. T. Shephard (1980) The effects of acetylcholine and ouabain on the sympathetic adrenergic neuroeffector junction in canine vascular smooth muscle. *Circ. Res.* 47: 845-854.
- Meyer, E. M., and J. R. Cooper (1981) Correlations between Na^+ - K^+ ATPase activity and acetylcholine release in rat cortical synaptosomes. *J. Neurochem.* 36: 467-475.
- Minchin, M. C. W. (1980) Veratrum alkaloids as transmitter-releasing agents. *J. Neurosci. Methods* 2: 111-121.
- Mulder, A. H., W. B. Van den Berg, and J. C. Stoof (1975) Calcium-dependent release of radiolabeled catecholamines and serotonin from rat brain synaptosomes in a superfusion system. *Brain Res.* 99: 419-424.
- Nakazato, Y., A. Ohga, and Y. Onoda (1978) The effect of ouabain on noradrenaline output from peripheral adrenergic neurones of isolated guinea pig vas deferens. *J. Physiol. (Lond.)* 278: 45-54.
- Nishimura, S., M. Sorimachi, and K. Yamagami (1981) Exocytotic secretion of catecholamines from the cat adrenal medulla by sodium deprivation: Involvement of calcium influx mechanism. *Br. J. Pharmacol.* 72: 305-317.
- O'Fallon, J. V., R. W. Brosemer, and J. W. Harding (1981) The Na^+ , K^+ ATPase: A plausible trigger for voltage-independent release of cytoplasmic neurotransmitters. *J. Neurochem.* 36: 369-378.
- O'Lague, P. H., D. D. Potter, and E. J. Furshpan (1978) Studies on rat sympathetic neurons developing in cell culture. I. Growth characteristics and electrophysiological properties. *Dev. Biol.* 67: 384-403.
- Palaty, V. (1981) Release of noradrenaline from the rat tail artery induced by inhibition of the sodium pump in calcium-free solution. *Can. J. Physiol. Pharmacol.* 59: 347-350.
- Paton, D. M. (1973) Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit atria. *Br. J. Pharmacol.* 49: 614-627.
- Patterson, P. H. (1978) Environmental determination of autonomic neurotransmitter function. *Annu. Rev. Neurosci.* 1: 1-17.
- Patterson, P. H., L. F. Reichardt, and L. L. Y. Chun (1976) Biochemical studies on the development of primary sympathetic neurons in cell culture. *Cold Spring Harbor Symp. Quant. Biol.* 40: 389-397.
- Pocock, G. (1983a) Ionic and metabolic requirements for stimulation of secretion by ouabain in bovine adrenal medullary cells. *Mol. Pharmacol.* 23: 671-680.
- Pocock, G. (1983b) Ion movements in isolated bovine adrenal medullary cells treated with ouabain. *Mol. Pharmacol.* 23: 681-697.
- Powis, D. A. (1983) Cardiac glycosides and autonomic neurotransmission. *J. Auton. Pharmacol.* 3: 127-154.
- Pressman, B. C. (1976) Biological applications of ionophores. *Annu. Rev. Biochem.* 45: 501-530.
- Pullman, M. E., H. S. Penefsky, A. Datta, and E. Racker (1960) Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble, dinitrophenol-stimulated adenosine triphosphatase. *J. Biol. Chem.* 235: 3322-3329.
- Raiteri, M., and G. Levi (1978) Release mechanisms for catecholamines and serotonin in synaptosomes. *Rev. Neurosci.* 3: 77-130.
- Raiteri, M., R. del Carmine, A. Bertolini, and G. Levi (1977) Effect of desmethylinipramine on the release of [3 H]-norepinephrine induced by various agents in hypothalamic synaptosomes. *Mol. Pharmacol.* 13: 746-758.
- Raiteri, M., F. Cerrito, A. M. Cervoni, and G. Levi (1979) Dopamine can be released by two mechanisms differentially affected by the dopamine transport inhibitor nomifensine. *J. Pharmacol. Exp. Ther.* 208: 195-202.
- Role, L. W., and R. L. Perlman (1983) Catecholamine uptake into isolated adrenal chromaffin cells: Inhibition of uptake by acetylcholine. *Neuroscience* 10: 987-996.
- Rutledge, C. O. (1978) Effect of metabolic inhibitors and ouabain on amphetamine- and potassium-induced release of biogenic amines from isolated brain tissue. *Biochem. Pharmacol.* 27: 511-516.
- Schwartz, A., G. E. Lindenmayer, and J. C. Allen (1975) The Na , K -ATPase: Pharmacological, physiological, and biochemical aspects. *Pharmacol. Rev.* 27: 3-134.
- Schwartz, E. A. (1982) Calcium-independent release of GABA from isolated horizontal cells of the toad retina. *J. Physiol. (Lond.)* 323: 211-217.
- Specht, S. C., and K. J. Sweadner (1984) Two different Na , K -ATPases in the optic nerve: Cells of origin and axonal transport. *Proc. Natl. Acad. Sci. U. S. A.* 81: 1234-1238.
- Sweadner, K. J. (1979) Two molecular forms of $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase in brain. Separation, and difference in affinity for strophanthidin. *J. Biol. Chem.* 254: 6060-6067.
- Sweadner, K. J. (1983) Post-translational modification and evoked release of two large surface proteins of sympathetic neurons. *J. Neurosci.* 3: 2504-2517.
- Vizi, E. S. (1972) Stimulation by inhibition of $(\text{Na}^+ - \text{K}^+ - \text{Mg}^{2+})$ -activated ATPase of acetylcholine release in cortical slices from rat brain. *J. Physiol. (Lond.)* 226: 95-117.
- Vizi, E. S., and F. Vyskocil (1979) Changes in total and quantal release of acetylcholine in the mouse diaphragm during activation and inhibition of membrane ATPase. *J. Physiol. (Lond.)* 286: 1-14.
- Vizi, E. S., T. Torok, A. Seregi, P. Serfozo, and V. Adam-Vizi (1982) Na , K -activated ATPase and the release of acetylcholine and noradrenaline. *J. Physiol. (Paris)* 78: 399-406.
- Vyas, S., and R. M. Marchbanks (1981) The effect of ouabain on the release of [^{14}C]-acetylcholine and other substances from synaptosomes. *J. Neurochem.* 37: 1467-1474.
- Wakade, A. R. (1981) Facilitation of secretion of catecholamines from rat and guinea pig adrenal glands in potassium-free medium or after ouabain. *J. Physiol. (Lond.)* 313: 481-498.
- Yazulla, S., and J. Kleinschmidt (1983) Carrier-mediated release of GABA from retinal horizontal cells. *Brain Res.* 263: 63-75.