A demonstration of sympathetic cotransmission

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Currently, most undergraduate textbooks that cover the autonomic nervous system retain the concept that autonomic nerves release either acetylcholine or norepinephrine. However, in recent years, a large volume of research has superseded this concept with one in which autonomic nerves normally release at least one cotransmitter along with a dominant transmitter that may or may not be acetylcholine or norepinephrine. Cotransmission involving the simultaneous release of norepinephrine, ATP, and neuropeptide Y can easily be demonstrated in an isometric ring preparation of the rat tail artery, which is described here. The experiment clearly demonstrates the principle of cotransmission but allows more advanced concepts in autonomic cotransmission to be addressed.

autonomic; norepinephrine; ATP; neuropeptide Y; isometric

ASPECTS OF TEACHING of the autonomic nervous system often retain a degree of dogma, depending on who is teaching the subject, their academic training and interest, and the type of students being taught. This is particularly true in the area of autonomic neurotransmission. Frequently, this is taught with reference to the notion of “one nerve, one transmitter,” with the repertoire of transmitter substances often being limited to only the classic transmitters of norepinephrine (NE) or ACh; if cotransmission is mentioned, it is often brief and vague (10, 13, 14). However, since the 1970s, a vast body of research has accumulated regarding how one nerve talks to another, spanning the synapse by the release of chemical messengers. Accumulation of this new knowledge has been particularly prolific in the area of autonomic transmission, where, in addition to the classic transmitters, many other nonadrenergic noncholinergic transmitters have now been found to be released with these classic transmitters (6). Indeed, cotransmission at autonomic neuroeffector junctions now appears to be the rule rather than the exception (19). In addition, there are complimentary numbers of receptor families and receptor subtypes that can mediate the actions of released cotransmitters. These actions can vary considerably in terms of the receptor subtype that the transmitter binds to and, therefore, the receptor-operated transduction mechanism for the transmitted signal. Furthermore, these receptors can be both postjunctional and presynaptic.

Thus, the autonomic neuroeffector junction is the site for considerable integration that potentially allows precise control over tissues innervated. This is a much more complicated arrangement than the simple input-output relationship at the autonomic neuroeffector junction that is widely taught in several areas of biomedical science (see a discussion, see Ref.

18). Yet, it is crucial for many current and potential medical treatments and research areas, as every transmitter-receptor combination offers a potential therapeutic target and research tool if suitable ligands are available or can be synthesized. Therefore, it is crucial that biomedical science students, particularly those in biomedical sciences, pharmacy, pharmacology, and medicine, have an appreciation of the concept of cotransmission.

Sympathetic control of the rat tail (or caudal ventral) artery (RTA) is a model that demonstrates several facets of cotransmission that can be easily demonstrated. There are three well-documented cotransmitters released on the activation of sympathetic innervation: 1) the classical transmitter NE, which in the case of the RTA acts predominantly on postsynaptic α₁- and α₂-adrenoceptors with time courses lasting tens of seconds (1, 2) and involves the recruitment of membrane-bound G proteins and second messenger pathways; 2) ATP, which mediates fast synaptic transmission (in seconds) via mainly postsynaptic P2X₁ receptors, which are composed of ligand-gated ion channels (16); and 3) neuropeptide Y (NPY), which, like many peptides, has a duration of action that can last several minutes and acts via NPY-Y₁ receptors (4, 5, 8, 20), although its natural actions in the RTA are to potentiate the actions of the other two transmitters rather than cause significant contraction directly (5).

All three components can readily be demonstrated in the RTA using a simple isometric contraction apparatus with the facility for field stimulation. This tissue has several advantages: it has a dense sympathetic innervation (23) and responds very readily to sympathetic nerve stimulation (5); it is a relatively homogeneous preparation [after removal of the endothelium, the only remaining interactions are between sympathetic postganglionic nerves and vascular smooth muscle (for a discussion, see Ref. 11)]; and, with careful dissection, it can yield many segments of vessel for isometric study. Thus, it can easily be used to demonstrate basic principles of cotransmission. This approach is currently being used to teach undergraduate medical students (20 students/group) in a “Student-Selected Component” module in their second year with the title of “Autonomic Control of the Cardiovascular System.” Students are formally lectured on the concept of autonomic cotransmission, and the practical is designed to reinforce that material. With the protocols outlined below, it fulfils the following objectives that are applicable to many basic undergraduate physiology/neuroscience modules:

• It demonstrates the influence of three cotransmitters at work in the RTA, reinforcing lecture material covering the occurrence of cotransmission in the autonomic nervous system;

• It allows students to undertake basic pharmacological protocols to examine the efficacy of transmitter agonists/antagonists and to assess their effects on electrically evoked sympathetic responses (by field stimulation);
It helps students to understand the need for control experiments with such an approach; and

- It allows students to make measurements from raw data traces, to analyze these data, and to draw conclusions as to the contribution of each transmitter to sympathetic responses.

Furthermore, it allows more subtle and advanced concepts of cotransmission to be explored (see DISCUSSION) that may be beyond the requirements of basic undergraduate courses, such as the following:

- Differential contribution of cotransmitters to evoked responses with different numbers/patterns of stimulation;
- Potential contributions from presynaptic receptors; and
- Potential problems with antagonist specificity.

For both practical and theoretical simplicity, the protocols outlined below are to demonstrate the roles of NE and ATP as cotransmitters. However, some less detailed suggestions for demonstrating the role of NPY are given in the DISCUSSION.

This preparation is particularly suited to fulfill these intentions as the protocols outlined here can be completed in 2–3 h, the results are usually visually obvious as they occur, and, in our experience, there are always aspects of the results that reinforce the teaching objective of demonstrating cotransmission. If results do not follow the expected outcomes, then this situation may be used to the benefit of the students by discussing the expected results, recognizing how their results differ from those expected, and what factors might account for any discrepancies.

MATERIALS AND METHODS

This practical is currently used as a demonstration for a group of up to 10 students/session, who can watch the experimental protocols being performed and observe the data by watching chart software on a computer screen. This is run as a demonstration in our hands rather than a practical that the students can participate in from start to finish as the dissection of the tail artery and mounting of vessel segments in the tissue bath requires a certain level of experience in microscope dissection that is often beyond the scope of many undergraduates, to guarantee responsive healthy tissue. However, if suitable technical assistance is at hand, and multiple setups are available, individual groups could perform the experiments themselves, provided that the tissue was mounted for them.

Equipment. The following equipment is needed:

- A standard tissue bath with two to four wells (well volume: 2–4 ml) with suction for fluid removal bubbled with 5% CO2–95% O2
- A water bath to warm the Krebs-Hansleit solution (see below) and a tissue bath to 37°C
- A peristaltic pump (2–4 lines)
- A laboratory interface/analog-to-digital converter
- A computer with suitable chart software
- Platinum wire stimulating electrodes (1 set/well) that can be positioned close to the vessel segments
- An electrical stimulator (0–40 V, variable pulse width and frequency)
- A standard dissecting microscope
- A cold light source
- A petri dish with a Sylgard base
- Dissection equipment, including a scalpel, blunt forceps, fine sharp forceps, and fine sharp spring-bow scissors

Kerbs’-Hansleit solution was composed of the following (in mM): 118.4 NaCl, 4.75 KCl, 25 NaHCO3, 1.19 KH2PO4, 1.18 MgSO4, 0.95 CaCl2, and 11.66 glucose.

Animals and dissection. The experimental procedures described below are all in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) and were approved by the Animal Welfare and Ethics Committee of Queen’s University. Tissue is removed from deceased male Sprague-Dawley rats (250–300 g, euthanized by an overdose of pentobarbitone or CO2 inhalation). The main RTA (the caudal ventral artery) lies in a ventral groove that can be accessed by first incising the skin of the uppermost ventral tail surface and then by cutting along the tough sheath that covers the ventral groove down the length of the tail using sharp fine scissors and forceps. The distal cut end of the vessel can then be lifted clear of the tail by fine forceps, cutting the connective tissue and connecting arteries between the RTA and the ventral groove, taking care not to overstretched the artery or allowing it to dry out at all. With the artery removed, segments of 3–5 mm in length can be cut under a dissecting microscope so that two small, fine stainless steel wire stirrups can be placed through the vessel lumen. These stirrups may be used to gently rub the inner surface of the vessel lumen to remove the endothelium before each ring is suspended in the tissue bath. The vessels must then be pretensed to 0.75 g and allowed to equilibrate for 1 h, with the vessel tension regularly readjusted to 0.75 g.

Protocols. The protocols assume that drugs are added to the tissue bath under “stop flow” conditions with the peristaltic pump switched off. Drug concentrations refer to final concentrations in the bath and are delivered directly into the bath via a pipette. Depending on the number of wells available, two identical protocols may be run in parallel, examining the role of either NE or ATP.

Nonreceptor-mediated contraction. At the beginning of the experiment, 60 mM KCl is added to cause receptor-independent contraction. This gives an early indication of the contractile state of the vessel segment, so that vessels with sluggish or small (<0.5 g) responses might be replaced. This is also conducted at the end of the experiment and allows judgement that any loss of a response to electrical stimulation is due to receptor antagonist rather than the demise of tissue condition.

Control electrical stimulation. Field stimulation of each vessel is delivered by the wire electrodes placed either side of each segment with a predetermined supramaximal voltage (up to 40 V) with a pulse width of 1 ms and a frequency of 20 Hz. A simple method for securing the position of the electrodes is to embed them in a lump of “plasticine” (~2 cm3) on the edge of each bath so that they can be fixed in a stable position close to the tissue. Stimuli are delivered in a battery of seven impulse numbers: 1, 2, 4, 8, 16, 32, and 100, with between 60 and 90 s in between separate bursts to allow recovery in between. This range of impulse numbers allows an examination of the relationship between nerve stimulation pattern/intensity with the contribution made by different cotransmitters (see Analysis of Results and DISCUSSION).

Response of vessel to agonist/antagonist. The addition of norepinephrine or purinergic agonists to a vessel demonstrates the presence of adrenoceptors [for example, the nonspecific adrenoceptor agonist NE (10−6 M) and purinoceptors [for example, the nonspecific P2X and P2Y receptor antagonist ATP (10−4 M)] on the vascular smooth muscle, although contraction does not necessarily prove that cotransmission using these two systems is present and active. After washout and recovery (~15 min for NE), antagonists can be applied. We use the nonspecific α1- and α2-adrenoceptor antagonist phentolamine (2 × 10−6 M) and the nonspecific P2 receptor antagonist suramin (10−4 M). These take ~5 and 30 min, respectively, to have their maximum effects. Finally, the same initial doses of the agonist can then be applied. If the antagonists are working, then the responses to the agonists should be blocked, confirming the efficacy of the antagonists in the next part of the protocol.

Sympathetically evoked responses in the presence of antagonists. The combined agonists and antagonists should then be washed out and
the antagonists reapplied. Their effects on sympathetically evoked responses can then be examined by delivering the same battery of stimuli. Once the battery has been delivered, the second antagonist can then be added, and the effects of the two antagonists present can be examined. It is likely that the responses are largely or completely abolished. Therefore, the final part of the protocol should be to reapply KCl. The response seen in the initial part of the protocol should be intact, allowing for a little tissue degradation over time.

Analysis of results. A representative trace (of genuine data) is shown in Fig. 1. Students should be instructed to calculate the absolute size of the response evoked by each electrical simulation by measuring the increase above the baseline tension (not zero tension), and both control (Fig. 1, A and B) and postantagonist (Fig. 1, C and D) responses should be plotted on the same graphs (Fig. 1, E and F). They should then calculate the response sizes after the antagonist as a percentage of the control (relative response). These two curves may then be plotted on the same graph (Fig. 1G). Students should present their own data or the communal data. However, if several data sets are available, mean data should also be generated and the students asked to compare their data with the mean data, as this often irons out any anomalous data points.

DISCUSSION

Having analyzed and presented the results, the students are then suitably equipped to address the following questions.

Do we have clear evidence of cotransmission? An advantage of the model used in this experiment is that there are significant contributions from both NE and ATP. Therefore, there should be significant reductions in the evoked responses in the presence of either the adrenergic or purinergic antagonists. It is reasonable to deduce that both transmitters must be released by sympathetic nerves simultaneously and contribute to the normal response evoked in the vessel. The reduction of the evoked response is most readily explained by each antagonist blocking their respective postjunctional receptors, so that a portion of the evoked response is removed. Assuming that some response is still left in the presence of one antagonist implies that a second cotransmitter is at work.

As assessed by adding adrenergic antagonists (phentolamine) first, what proportion of the response is due to ATP? As assessed by adding purinergic antagonist (suramin) first, what proportion of the response is due to NE? Is there any evidence of variation of the contributions of NE or ATP depending on the number of stimuli? These questions are asked in such a way as to allow the students to develop an appreciation of the phenomenon of synergy that is known to exist between the actions of NE and ATP (21) and also to appreciate that stimuli of different impulse numbers can evoke greater responses.

Fig. 1. A and B: control responses evoked by electrical stimulation of sympathetic nerves to the rat tail artery with 1–100 impulses. C and D: responses in the presence of the antagonists phentolamine (2 × 10⁻⁶ M; C) and suramin (10⁻⁴ M; D). E and F: absolute peak responses for controls (●) and in the presence of phentolamine (E; ■ and dashed line) or suramin (F; ● and dashed line). G: relative responses in the presence of phentolamine (● and solid line) or suramin (■ and dashed line) expressed as a percentage of the control.
mediated by one cotransmitter compared with another (see Refs. 5 and 25). For the data shown in Fig. 1, in the presence of phentolamine, the remaining purinergic contribution would account for ~35% of the response for 2 impulses, as opposed to ~10% of the response to 100 impulses. Thus, from this protocol alone, there is evidence of a differential contribution of cotransmitters depending on impulse numbers (or the pattern/strength of the stimulus), with ATP being more important at lower impulses and NE contributing more at higher impulse numbers. In the presence of suramin, a similar story emerges as it would appear that ~15% of the response to 2 impulses is due to NE and ~55% of the response to 100 impulses. Again, this implies that ATP makes a greater contribution to the response evoked by lower impulse numbers, and NE contributes more as impulse number increases. However, for both 2 and 100 impulses, the combined individual responses (50% and 60%, respectively) are less than the control response with no antagonists present. Thus, we have evidence that the two transmitters acting together have greater action compared with the added actions of each transmitter considered separately, i.e., the two cotransmitters are acting in synergy.

These phenomena have been well documented in the literature, although there is little known about the mechanisms by which they arise. There is evidence that as the impulse numbers increase there is an accumulation of NE in the junction as the clearance mechanisms become saturated (1, 24), and, therefore, NE has a greater influence under these circumstances. Conversely, the fast nature of P2X1 receptor coupling implies that ATP makes a greater contribution to the response evoked by lower impulse numbers, and NE contributes more as impulse number increases. However, for both 2 and 100 impulses, the combined individual responses (50% and 60%, respectively) are less than the control response with no antagonists present. Thus, we have evidence that the two transmitters acting together have greater action compared with the added actions of each transmitter considered separately, i.e., the two cotransmitters are acting in synergy.

A relatively frequent variation in these results is seen in the presence of suramin, as the evoked responses may sometimes potentiate (5, 12). In this circumstance, it is possible that the potentiation results from the greater release of transmitter due to antagonism of presynaptic inhibitory P2X receptors (3).

The presence of the second antagonist on top of the first often abolishes all remaining responses, which again confirms the actions of two cotransmitters. If there are any responses remaining in the presence of two antagonists, then that may be evidence for a third cotransmitter. This can be addressed by the following question.

If there was a residual response to electrical stimulation after NE and ATP blockade, what could it be and how would you investigate it? In our experience, the application of ligands for NPY is less consistent. The direct contractile effect of the peptide is only apparent at high concentration (e.g., $10^{-6}$ M), but not reliably. However, potentiation of electrically evoked responses is readily apparent at lower concentrations of NPY (e.g., $7.5 \times 10^{-8}$ M; see below), as is inhibition with BIBP-3226 ($10^{-5}$ M), particularly when administered during trains of moderate stimulation (e.g., 5 impulses at 20 Hz, every 60 s (see Ref. 5)). Thus, a contribution from a third cotransmitter can be demonstrated relatively simply. However, the contribution from NPY is much more variable. The reason for this variability is not that apparent. It may be due to variation in NPY contributions between animals. But knowing that segments from the same artery can show marked variation in NPY contributions, there must be an element of variation in the way in which tissue is handled during dissection, mounting, etc., which is, to some extent, unavoidable. The remaining responses may also be due to insufficient blockade of the other two cotransmitter systems.

In addition to the specific issues relating to sympathetic cotransmission, more general issues relating to this type of experiment may be asked, as in the question below.

What assumptions are made during these protocols? How could you test them? Students should be prompted to examine the assumptions that may not have been thought of up to this point. These may include the following:

- The assumption that the ligands used are receptor specific and some consideration of the specificity, or otherwise, associated with certain agonists/antagonists. This could also include pharmacological strategies for receptor characterization, including agonist profiling.
- Direct stimulation of smooth muscle (which is unlikely with these stimulation parameters) could be discounted if the evoked response could be blocked in the presence of agents that inhibit neuronal conduction (such as tetrodotoxin, a fast Na+ channel blocker, the effects of which are relatively permanent) or inhibition of the sympathetic neuroeffector junction (such as guanethidine, which has multiple synaptic effects but can take >45 min to work).
- It is assumed that when antagonists are applied, they are working in full; however, if responses remain in the presence of antagonists, it is possible that this may be due to the antagonist effect declining, etc. Therefore, such protocols rely on the actions of well-characterized ligands.
- The vascular endothelium makes no contribution to the responses evoked by exogenous ligands or electrical stimulation. This could be tested by running the protocols in endothelium-denuded vessels, which is provided by gently rubbing the inner lumen of each vessel segment with the metal hooks. Incidentally, this preparation is entirely suitable for demonstrating some functional aspects of the endothelium, such as ACh/nitric oxide-mediated vasodilatation.
- The concentrations of agonists used in the first part of the protocol are maximal. This is necessary to ensure that the action of the antagonists completely blocks the actions of sympathetically released transmitters, allowing the conclusion that any residual response is due to another cotransmitter.

What are the advantages and limitations of this preparation? This question should allow students to explore the advantages and disadvantages of in vitro preparations, compared with, say, in vivo experiments. This could include a consideration of control of external factors and problems with interpretation of function in the whole animal from in vitro experiments. A discussion of the advantages (such as physiological relevance) and disadvantages (such as lack of control or legislation for in vivo work) for in vivo preparations could also be considered.

Conclusions. This practical/demonstration can fulfil the student’s educational needs on several levels. Specific aspects of cotransmission are emphasized to students by direct observation of evidence for its existence. This occurs during the
experimental procedure as the effects of the pharmacological interventions are usually visually obvious. This is then reinforced by the process of analyzing the results that they have gained and quantifying the cotransmitter contributions. Students are then in a position to engage with more advanced concepts in autonomic neuroscience such as cotransmitter synergism and the influence of sympathetic discharge patternning on cotransmitter contributions. This is an effective way to alter existing “mental models” that students may have about cotransmission (17).

From a more general viewpoint, students experience several transferable skill activities. They are required to understand the technical aspects of the experimental setup to appreciate the actual end point being measured and analyzed (in this case, isometric tension of the vessels). They must also go through the process of extracting data from the raw traces. Both of these processes give a much broader appreciation of how scientific experiments allow gaps in theoretical knowledge to be spanned in addition the primary objective of teaching the principle of cotransmission. It also provides students with some insight into the scientific process and the need for experimental controls to allow objective assessment of the results. This insight extends to the nature of scientific experiments and biological variation, due to the fact that not every experiment yields the same or expected results. This approach to teaching also fosters the “desire to learn” (22). All of these benefits would be very hard to replicate in any form other than observing/participating in actual experiments.

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DISCLOSURES

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