A demonstration of sympathetic cotransmission

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Johnson CD. A demonstration of sympathetic cotransmission. Adv Physiol Educ 34: 217–221, 2010; doi:10.1152/advan.00070.2010.—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 However, in recent years, a large volume of research has superseded this concept with one in which autonomic nerves normally release at least one cotransmitter along with a dominant transmitter that may or may not be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 prejunctional.

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 $\alpha_1$- and $\alpha_2$-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 postjunctional $P2X_1$ receptors, which are comprised 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-Y$_1$ 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 fulfills 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);

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• 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 fulfil 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 segmental vessels 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
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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 iron's 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 (10⁻⁷ 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 number increases 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 with smooth muscle contraction due to activation of ion channels, and the reduced per-pulse release of ATP as the number of impulses increases (1, 24), may account for the greater influence of ATP at lower impulse numbers. This is in accordance with an emerging realization that patterning aspects of sympathetic discharge can have profound effects on the contributions made by cotransmitters and that intermittent patterns are more effective at evoking vascular responses on a per-pulse basis (7, 9, 15).

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 a 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 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. The 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 fulfill 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 patterning 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

No conflicts of interest, financial or otherwise, are declared by the author(s).

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