

Letters to the Editor: Cardiomyocyte-secreted acetylcholine

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I READ THE paper, “Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart” (1), on acetylcholine (ACh) originating in cardiac myocytes, with great interest. The significance of this phenomenon continues to fascinate since it was first described over 40 years ago. The authors have overlooked reports and experimental limits from the earlier papers that deserve careful consideration.

Edouard Coraboeuf and colleagues (2) reported in 1970 that cardiac myocytes could synthesize and release ACh. Of note, they obtained data from embryonic hearts before innervation occurred. They also observed that electrical stimulation of the embryonic chick heart caused a transient slowing of heart rate and an increased release of ACh (2). The transient slowing was opposed by atropine. So far, more recent reports (1, 3) have not asked whether stimuli can regulate ACh secretion.

Divergent results on the age of animals whose heart cells can secrete ACh are another unsettled issue. You report that myocytes from neonatal mice secrete ACh. This is opposite the report by Rana *et al.* (3), who state that “. . . adult, but not neonatal cardiomyocytes are able to synthesize, transport and excrete acetylcholine in the rat heart.” This discrepancy calls for an explanation, inasmuch as rats and mice develop similarly after nearly equal gestation periods.

The immunofluorescent detection of choline acetyltransferase (ChAT) requires validation of specificity. Some years ago (4), it was reported that the method to detect ChAT must carefully distinguish ChAT from carnitine acetyltransferase, which is found in cardiac muscle. Thus, Roskoski *et al.* (4) showed that embryonic chick heart cells, *per se*, had no ChAT activity and no neurons. Neonatal rat heart cells in culture had no ChAT, but carnitine acetyltransferase was present. Thus, in the absence of cholinergic neurons, heart cells did not synthesize ACh (4). Does the immunofluorescent method you use distinguish between ChAT and carnitine acetyltransferase?

The experiments with carbachol and pyridostigmine overlook important drug properties. It is re-

ported that addition of carbachol caused ACh release from cardiac myocytes, as indicated by increased NO production, a downstream fluorescent signal (1). There are concerns with this observation. First, cholinergic neurons have muscarinic receptors whose occupancy by ACh reduces ACh release (5). This negative-feedback mechanism, a physiological hallmark of homeostasis, seems lacking in your system. Second, “. . . pyridostigmine also augmented the fluorescent signal (Supplemental Fig. S2F).” Pyridostigmine, like neostigmine (and carbachol), has a quaternary N that not only renders it impermeable to cell membranes but also makes it an agonist acting directly at cholinergic sites, just like carbachol. Thus, pyridostigmine and neostigmine can activate receptors, in addition to inhibiting acetylcholinesterase. Similarly, hemicholinium-3 (HC-3) is another quaternary N compound whose actions against pyridostigmine can be explained by HC-3 binding to the same site as pyridostigmine and occluding its effect. Indeed, one should test vesamicol and HC-3 against carbachol, as was done against pyridostigmine in Supplemental Fig. 2SF. Such testing should clarify some essential elements of the hypothesis.

The current state of the problem seems not to have evolved significantly from that described by earlier investigators. This is a consequence, in part, of a failure to attend to precedent. EJ

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Response by Ashbeel Roy, William C. Fields, Cibele Rocha-Resende, Rodrigo R. Resende, Silvia Guatimosim, Vania F. Prado, Robert Gros, Marco A. M. Prado²

THE STUDY BY Coraboeuf *et al.* (1) described an acetylcholine (ACh)-like substance of myocardial origin that was secreted from chicken hearts and induced contraction of the leech dorsal muscle. The concept of ACh secretion from cardiac tissue existed even prior to this study. Briscoe and Burn (2) published a report in 1954, wherein they described the release of an ACh-like substance through the use of various biological assays. However, none of these studies showed that the ACh-like material was, in fact, ACh. It is only recently that studies by our group and others have independently provided evidence for a molecular mechanism by which ACh can be secreted, by demonstrating the presence of the machinery required to synthesize and secrete ACh in mammalian cardiomyocytes. We had no intention of overlooking these early reported experiments; however, because of their technical limitations, we cited the more recent manuscripts that provided mechanistic insight (3–6).

With respect to the manuscript that reported an absence of cholinergic machinery in neonatal rat cardiomyocytes (5), we would like to point out that this is likely a result of technical differences. We have previously published a manuscript (3) wherein we characterized the importance of the intrinsic cholinergic system *in vitro*. In this previous study, we reported that both neonatal and adult rodent cardiomyocytes express prototypical markers of the cholinergic system. Furthermore, Kakinuma *et al.* (4) reported a similar finding previously, as they positively identified these markers in both neonatal and adult rat cardiomyocytes. Hence, two manuscripts reported the presence of cholinergic machinery in rodent neonatal cardiomyocytes, whereas one manuscript did not. Additionally, our manuscript in *The FASEB Journal*, to the best of our knowledge, is the first to demonstrate vesicular ACh transporter (VACHT)-dependent ACh release from neonatal cardiomyocytes. As such, technical differences may explain the different results from Rana *et al.* (5).

The manuscript by Roskoski *et al.* (7), mentioned by

Dr. Pappano, did not examine immunoreactivity but rather, investigated choline acetyltransferase (ChAT) activity and carnitine acetyltransferase activity in chicken cardiomyocytes. The commercial antibodies used in our *FASEB J.* manuscript have been used previously by several laboratories and shown to be specific for ChAT (8–10). This antibody was also validated in our previous publication (3). Therefore, there is no reason to infer that the antibody is non-specific. Additionally, in our *FASEB J.* study, we present genetic evidence that knockout of ChAT exclusively in cardiomyocytes has functional consequences, supporting not only the presence of ChAT but also a functional role for ACh derived from cardiomyocytes.

It is important to note that carbachol was used as an agonist for muscarinic receptors in our experiment and thus, served as a positive control for muscarinic receptor activation and NO production. We do not argue that carbachol induces ACh release, and Dr. Pappano may have misunderstood the assay. Moreover, the rationale that carbachol causes presynaptic inhibition in neurons and, as such, should do the same in cardiomyocytes is misleading. There are examples in which presynaptic muscarinic activation can increase secretion of neurotransmitters (11–13). Our work has demonstrated the physiological relevance of myocyte-derived ACh secretion *in vivo*, and we can now investigate the mechanisms regulating this release. Regarding the direct actions of pyridostigmine (or hemicholinium-3 or vesamicol) on muscarinic receptors, we would ask Dr. Pappano to refer to our previous publication (3), in which we validated this assay using several different methods. The most relevant validation is found in our current manuscript. Pyridostigmine cannot activate NO production in cardiomyocytes in the absence of VACHT. If the drug were activating muscarinic receptors directly, it should have increased NO in cardiomyocytes from conditional knockout mice. Furthermore, diaminofluorescein fluorescence and NO production were used in this study as an indirect method of measuring ACh secretion in cardiomyocytes. In addition, we have used both a fluorometric assay for ACh as well as HPLC with electrochemical detection to confirm ACh secretion from cardiomyocytes. Therefore, we validated ACh secretion from cardiomyocytes using three distinct methods, only one of which is a bioassay.

We strongly disagree with the statement by Dr. Pappano that the field has not evolved significantly over the past years. Only through the use of molecular genetics can earlier observations advance from curiosity and a potentially *in vitro* phenomenon to a physiologically relevant mechanism. Hence, the field has moved forward significantly by defining the presence of neuronal machinery in cardiomyocytes and examining its relevance in heart function *in vivo*.

In addition to our report, similar molecular techniques have provided evidence for a role of non-neuronal ACh in other systems. For example, it has been shown recently that lymphocytes produce and secrete ACh to regulate the cholinergic anti-inflamma-

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