3. Rana, O. R., Schauerte, P., Kluttig, R., Schröder, J. W., Koenen, R. R., Weber, C., Nolte, K. W., Weis, J., Hoffmann, R., Marx, N., and Saygili, E. (2010) Aceytlcholine as a age-dependent non-neuronal source in the heart. *Auton. Neurosci.* **156,** 82–89
4. Roskoski Jr., R., McDonald, R. I., Roskoski, L. M., Marvin, W. J., and Hermsmeyer, K. (1977) Choline acetyltransferase activity in heart: evidence for neuronal and not myocardial origin. *Am. J. Physiol.* **233,** H642–H646
5. Wellstein, A., and Pitschner, H. F. (1988) Complex dose-response curves of atropine in man explained by different functions of M1- and M2-cholinoceptors. *Naunyn Schmiedebergs Arch. Pharmacol.* **338,** 19–27

Response by Ashbeel Roy, William C. Fields, Cibele Rocha-Resende, Rodrigo R. Resende, Silvia Guatimosim, Vania F. Prado, Robert Gros, Marco A. M. Prado[2]

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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tory pathway (14). Moreover, pancreatic α cells can also secrete ACh to regulate insulin secretion in humans (15). Thanks to modern molecular techniques, we will gain a detailed understanding of the relevance of non-neuronal cholinergic function in different tissues in the near future (16). FJ

REFERENCES

1. Coraboeuf, E., LeDouarin, G., and Obrecht-Coutris, G. (1970) Release of acetylcholine by chick embryo heart before innervation. *J. Physiol.* **206,** 383–395
2. Briscoe, S., and Burn, J. H. (1954) The formation of an acetylcholine-like substance of the isolated rabbit heart. *J. Physiol.* **126,** 181–190
3. Rocha-Resende, C., Roy, A., Resende, R., Ladeira, M. S., Lara, A., de Morais Gomes, E. R., Prado, V. F., Gros, R., Guatimosim, C., Prado, M. A., and Guatimosim, S. (2012) Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. *J. Mol. Cell. Cardiol.* **53,** 206–216
4. Kakinuma, Y., Akiyama, T., and Sato, T. (2009) Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. *FEBS J.* **276,** 5111–5125
5. Rana, O. R., Schauerte, P., Kluttig, R., Schroder, J. W., Koenen, R. R., Weber, C., Nolte, K. W., Weis, J., Hoffmann, R., Marx, N., and Saygili, E. (2010) Acetylcholine as an age-dependent non-neuronal source in the heart. *Auton. Neurosci.* **156,** 82–89
6. Roy, A., Fields, W. C., Rocha-Resende, C., Resende, R. R., Guatimosim, S., Prado, V. F., Gros, R., and Prado, M. A. (2013) Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. *FASEB J.* article fj. 13-238279 [published online September 9]
7. Roskoski, Jr., R., McDonald, R. I., Roskoski, L. M., Marvin, W. J., and Hermsmeyer, K. (1977) Choline acetyltransferase activity in heart: evidence for neuronal and not myocardial origin. *Am. J. Physiol.* **233,** H642–H646
8. Sharott, A., Doig, N. M., Mallet, N., and Magill, P. J. (2012) Relationships between the firing of identified striatal interneurons and spontaneous and driven cortical activities in vivo. *J. Neurosci.* **32,** 13221–13236
9. Nguyen, B. L., Li, H. M., Fishbein, M. C., Lin, S. F., Gaudio, C., Chen, P. S., and Chen, L. S. (2012) Acute myocardial infarction induces bilateral stellate ganglia neural remodeling in rabbits. *Cardiovasc. Pathol.* **21,** 143–148
10. Borodinsky, L. N., Root, C. M., Cronin, J. A., Sann, S. B., Gu, X. N., and Spitzer, N. C. (2004) Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. *Nature* **429,** 523–530
11. Xu, M., Mizobe, F., Yamamoto, T., and Kato, T. (1989) Differential effects of M1- and M2-muscarinic drugs on striatal dopamine release and metabolism in freely moving rats. *Brain Res.* **495,** 232–242
12. De Klippel, N., Sarre, S., Ebinger, G., and Michotte, Y. (1993) Effect of M1- and M2-muscarinic drugs on striatal dopamine release and metabolism: an in vivo microdialysis study comparing normal and 6-hydroxydopamine-lesioned rats. *Brain Res.* **630,** 57–64
13. Guo, J. Z., and Chiappinelli, V. A. (2000) Muscarinic receptors mediate enhancement of spontaneous GABA release in the chick brain. *Neuroscience* **95,** 273–282
14. Rosas-Ballina, M., Olofsson, P. S., Ochani, M., Valdes-Ferrer, S. I., Levine, Y. A., Reardon, C., Tusche, M. W., Pavlov, V. A., Andersson, U., Chavan, S., Mak, T. W., and Tracey, K. J. (2011) Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* **334,** 98–101
15. Rodriguez-Diaz, R., Dando, R., Jacques-Silva, M. C., Fachado, A., Molina, J., Abdulreda, M. H., Ricordi, C., Roper, S. D., Berggren, P. O., and Caicedo, A. (2011) α Cells secrete acetylcholine as a non-neuronal paracrine signal priming β cell function in humans. *Nat. Med.* **17,** 888–892
16. Prado, V. F., Roy, A., Kolisnyk, B., Gros, R., and Prado, M. A. (2013) Regulation of cholinergic activity by the vesicular acetylcholine transporter. *Biochem. J.* **450,** 265–274