1382–1384 *Nucleic Acids Research, 1996, Vol. 24, No. 7* *© 1996 Oxford University Press*

A novel method of identifying living transfected cardiac myocyte

Bruce T. Liang

Department of Medicine, Cardiovascular Division, University of Pennsylvania Medical Center, 504 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104, USA

Received December 7, 1995; Revised and Accepted February 21, 1996

Transfection of DNA into cardiac myocytes provides an excellent opportunity to study the function and regulation of cardiac proteins. Because of the lack of an immortal cardiac cell line, selection of stably transfected cardiac cell has not been possible. Transient transfection of primary cultures of cardiac cells is associated with relatively low and variable efficiency of gene transfer. While infection of cultured cardiac myocytes by adenovirus can result in high-efficiency gene transfer (1), preparation of the viral construct is time-consuming and tedious, slowing studies on the effect of gene transfer. Further, the effects of virus on cardiac cells need to be established before studies on the transgene in infected cardiac cells can be interpreted. Method on identifying live transfected myocytes has been lacking. In the current study, a simple and rapid approach that allows identification of living transfected cardiac myocytes has been developed.

Ventricular cells cultured from chick embryos 14 days *in ovo* were used as the cardiac model to test this new method (2). Cultured cardiac cells were transfected with an expression vector encoding the neomycin-resistance gene using the previously described modification of the calcium phosphate precipitates method (3), allowed to recover for 12 h, and then exposed to 30 μg/ml of G418 until the beginning of the study, 24 h later. The vector, $pcDNA_3$ (Invitrogen), expresses an aminoglycoside phosphotransferase gene and contains a multiple cloning region which allows subcloning and expression of the gene of interest (4,5). To facilitate identification of transfected myocytes, Lac Z cDNA was inserted into the multiple cloning region of $pcDNA_3$ ($pcDNA_3$/Lac Z). Myocyte transfected with $pcDNA_3$/Lac Z should express both β-galactosidase and aminoglycoside phosphotransferase. This was tested as follows. The presence of G418 following exposure of myocytes to $pcDNA_3$/Lac Z and calcium phosphate distinguished two groups of myocytes. The first group exhibited only a slight increase in the basal contractile amplitude in response to 2.4 mM extracellular calcium (calcium-unresponsive) whereas a second group of myocytes showed a much more pronounced calcium-induced increase in contractile amplitude (calcium-responsive) (Table 2 and Fig. 1). The extent of calcium-induced increase in contractility in the calcium-responsive myocytes was similar to the extent of increase found in myocytes exposed to $pcDNA_3$/Lac Z without a subsequent exposure to G418 (41.4 ± 10%, Table 2) or in myocytes not subjected to any DNA–calcium phosphate precipitates (43 ± 6%, n = 14). Such data are consistent with the notion that the calcium-responsive myocytes, which exhibited a normal calcium-induced increase in contractile amplitude, represented transfected myocytes expressing the neomycin-resistance gene. To further test this notion, myocytes were cultured on specially letter- and number-coded coverslips (Cellocate, Eppendorf), which facilitated identification of transfected myocytes. The contractile response of myocytes transfected with $pcDNA_3$/Lac Z can be identified following determination of the contractility response by the β-galactosidase chromogenic substrate X-gal (5-bromo-4-chloro-indolyl-β-galactopyranoside). Fourteen myocytes showed a 43.8 ± 3.1% increase in the contractile amplitude above the basal level; 12 (86%) of these myocytes were positive for X-gal. Fifteen myocytes exhibited only a 6 ± 2% increase in response to 2.4 mM external Ca^{2+}. Thirteen of these 15 myocytes (87%) were negative for X-gal. Thus, calcium responsiveness is highly predictive of transfection with a positive predictive value of 86% and a negative predictive value of 87%. Such data suggest that myocytes transfected with $pcDNA_3$/Lac Z expressed both the Lac Z and the aminophosphotransferase genes.

Table 2. Effects of transfecting $pcDNA_3$/Lac Z on myocyte responsiveness to increased extracellular calcium concentration

Exposed to $pcDNA_3$/Lac Z		Basal amplitude (μm)	Percent increase with 2.4 mM Ca^{2+}
With G418	Group 1	0.61 ± 0.16	7.9 ± 3
	Group 2	0.74 ± 0.11	45 ± 8
Without G418		0.78 ± 0.15	41.4 ± 10

Cultured ventricular myocytes were transfected with 20 μg $pcDNA_3$/Lac Z by a modified calcium phosphate precipitates method, allowed to recover and then incubated in the presence or absence of G418 (30 μg/ml) for 24 h. After achieving steady state in the presence of 0.9 mM extracellular calcium, myocytes were exposed to medium containing 2.4 mM calcium and the increase in contractile amplitude determined as percent increase above the basal contractile amplitude. Data represented mean± SE of 17 myocytes for group 1, 19 myocytes for group 2 and 15 myocytes for the group that was exposed to $pcDNA_3$/Lac Z but not to G418.

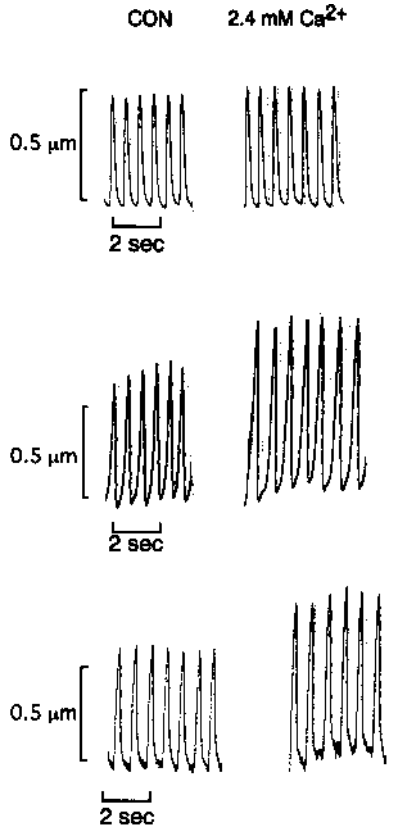

Figure 1. The presence of G418 following exposure of cardiac myocytes to pcDNA$_3$/Lac Z and calcium phosphate distinguishes calcium-responsive from calcium-unresponsive myocvtes. Cultured ventricular myocytes were exposed to 20 μg pcDNA$_3$/Lac Z and calcium phosphate, which was followed by treatment with or without G418 (30 μg/ml). The responsiveness of myocyte to 2.4 mM extracellular calcium was determined as described in the footnote to Table 1. The upper tracing was the contractile response of a myocyte to 2.4 mM Ca^{2+} that was typical of the G418-treated myocytes exhibiting a weak calcium-induced positive inotropic response (group 1). The tracing was representative of 16 others. The middle tracing was the inotropic response of myocyte that was typical of the G418-treated myocytes which showed a strong positive inotropic response (group 2). The tracing was typical of 18 others. The lower tracing represented the inotropic response of myocyte that was exposed to pcDNA$_3$/Lac Z–calcium phosphate but was not exposed to G418. The tracing was typical of 14 others.

In using this method to find living transfected myocytes, myocytes were exposed to 2.4 mM calcium first and the calcium-responsive myocytes, representing the G418-resistant transfected myocytes, were then identified. Because nearly half of the myocytes were calcium responsive, the method allowed rapid identification of transfected myocytes. Transfected myocytes can then be used to study the contractile effect mediated by protein expressed from foreign DNA. An example of this is illustrated in Figure 2. Myocytes were transfected with rat cDNA encoding the stimulatory adenosine A_{2a} receptor (kindly provided by Dr S. Reppert, pcDNA$_3$/A_{2a}R) followed by G418 exposure. Activation of the A_{2a} receptor normally causes an increase in myocyte contractility (6). Calcium-responsive myocytes, representing transfected myocytes overexpressing the A_{2a} receptor, showed a much larger increase in contractile amplitude in response to the A_{2a} receptor-selective agonist CGS21680 {2-[4-(2carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine} (percent increase = 29 ± 3%, n = 12, ± SE) than

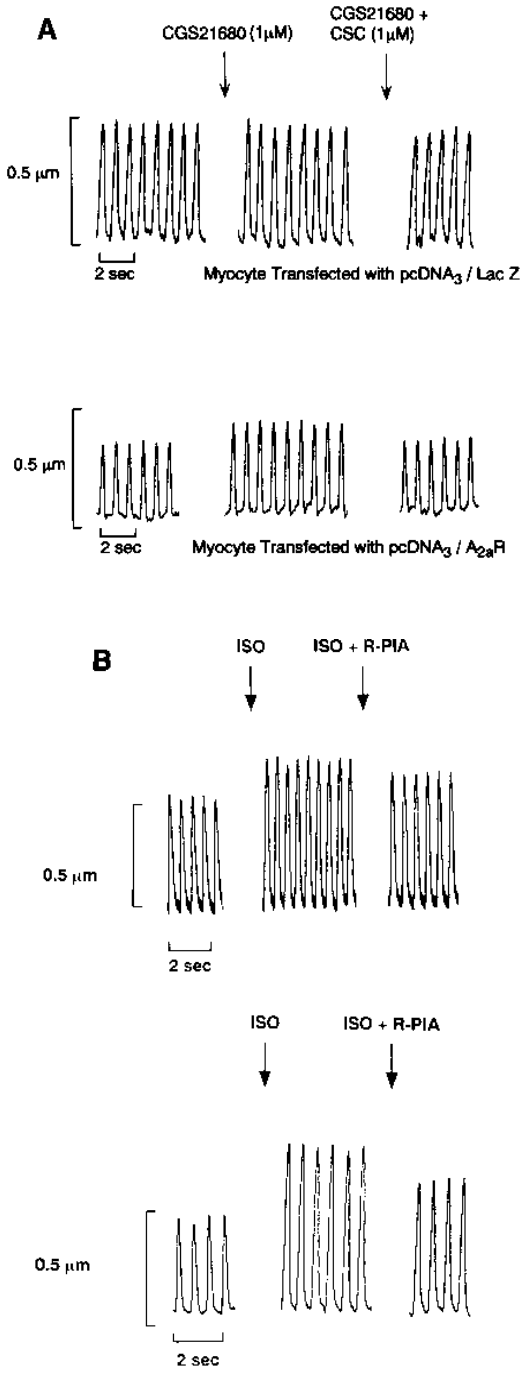

Figure 2. Inotropic response of myocytes transfected with DNAs encoding the stimulatory adenosine A_{2a} receptor and the inhibitory A_1 receptor. Ventricular myocytes were cultured, transfected with 20 μg of pcDNA$_3$/Lac Z, pCDNA$_3$/A_{2a}R or pcDNA$_3$/A_1AR, and then exposed to G418 prior to the contractility measurement as described in the footnote to Table 1. (**A**) In myocytes transfected with pCDNA$_3$/A_{2a}AR, calcium-responsive myocytes were exposed to 1 μM CGS21680 and then to CGS21680 plus CSC (1 μM). The tracings were typical of nine myocytes from three cultures transfected with pcDNA$_3$/Lac Z (upper tracing) and eight myocytes from three other cultures transfected with pCDNA$_3$/A_{2a}R (lower tracing). (**B**) In myocytes transfected with pcDNA$_3$/A_1AR, the calcium-responsive myocytes were exposed to 0.3 μM isoproterenol and then to isoproterenol plus the A_1 receptor agonist R-PIA (10 μM). Tracings were typical of 11 myocytes from three cultures transfected with pcDNA$_3$/Lac Z (upper tracing) and 12 myocytes from four cultures transfected with pcDNA$_3$/A_1AR (lower tracing).

calcium-responsive myocytes transfected with pcDNA$_3$/Lac Z (percent increase = 14 ± 1.5%, n = 11; P < 0.01, t-test) (Fig. 2A). The CGS21680-stimulated increase in contractility was completely blocked by the A_{2a} receptor-selective antagonist CSC [8(chloro-styryl)caffeine]. On the other hand, calcium-unresponsive myocytes, representing untransfected myocytes, did not show any increase in contractile amplitude in response to CGS21680 (not shown). Myocytes transfected with A_{2a} receptor cDNA also showed a more pronounced CGS21680-stimulated cAMP level. The increase above basal was 41 ± 3.5 pmol cAMP/mg protein for myocytes transfected with pcDNA$_3$/A_{2a}R (± SE, n = 4) versus 21 ± 2.3 pmol/mg for myocytes transfected with pcDNA$_3$/Lac Z (n = 4, P < 0.01, t-test). The data indicate the expression of exogenous A_{2a}R cDNA into a functional protein whose stimulatory contractile effect can be determined in the transfected myocyte. If the effect of protein expressed from foreign DNA is to inhibit myocyte contractility, the question arises regarding whether such inhibitory effect in the transfected myocyte can also be determined. To test this question, myocytes were transfected with rat cDNA encoding the inhibitory adenosine A_1 receptor (pcDNA$_3$/A_1AR). Myocytes transfected with pcDNA$_3$/A_1AR remained calcium-responsive with a calcium-induced increase in contractile amplitude similar to that determined in myocytes transfected with pcDNA$_3$/Lac Z (not shown). Activation of A_1 receptor normally induces an inhibition of isoproterenol-stimulated contractility. Myocytes transfected with the A_1 receptor DNA exhibited a further inhibition of the isoproterenol-induced stimulation of contractility in response to the A_1 receptor agonist N^6-R-phenyl-2-propyladenosine (R-PIA) (percent inhibition = 28.8 ± 1.1%, n = 12, ± SE) compared with myocytes transfected with pcDNA$_3$/Lac Z (percent inhibition = 15.8 ± 0.8%, n = 11) (Fig. 2B). Calcium-responsive myocytes overexpressing the A_1 receptor also exhibited an inhibition of the basal contractile amplitude during A_1 receptor stimulation (percent inhibition = 14 ± 1.1%, n = 12) whereas calcium-responsive myocytes transfected with pcDNA3/Lac Z showed no such inhibition. Calcium-unresponsive myocytes failed to show any inhibitory contractile response to R-PIA (data not shown). Thus, selection of G418-resistant, calcium-responsive myocytes allowed determination of the inhibitory contractile effect mediated by the exogenous A_1 receptor DNA.

Taken together, the current method enables rapid and reliable identification and determination of contractile behavior in live transfected cardiac myocytes. The development of this novel method should be useful in studies of the expression and contractile function of exogenous DNA in the cardiac myocyte.

ACKNOWLEDGEMENTS

This work was supported by RO1–HL48225 from the National Institutes of Health and an Established Investigatorship from the American Heart Association.

REFERENCES

1 Kirshenbaum,L.A., MacLellan,W.R., Mazur,W., French,B.A. and Schneider,M.D. (1993) *J. Clin. Invest.*, **92**, 381–387.
2 Liang,B.T. and Galper,J.B. (1988) *Biochem. Pharmacol.*, **37**, 4549–4555.
3 Xu,H., Miller,J. and Liang,B.T. (1992) *Nucleic Acids Res.*, **20**, 6425–6426.
4 Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **l**, 327–341.
5 Boshart,M., Weber,F., Jahn,G., Dorsch-Hasler,K., Fleckenstein,B. and Schaffner,W. (1985) *Cell*, **41**, 521–530.
6 Liang,B.T. and Haltiwanger,B. (1995) *Circ. Res.*, **76**, 242–251.