Reduction in Intracellular Calcium Levels Inhibits Myoblast Differentiation*

George A. Porter, Jr.,§, Ryan F. Makuck‡, and Scott A. Rivkees¶||

From the Department of Pediatrics, Divisions of §Cardiology and ¶Endocrinology, Yale University School of Medicine, New Haven, Connecticut 06520

In myocytes, calcium plays an important role in intracellular signaling and contraction. However, the ability of calcium to modulate the differentiation of striated muscle cells is poorly understood. To examine this issue we studied C2C12 cells, which is a myoblast cell line that differentiates in vitro. First, we observed that the L-type calcium channel blockers nifedipine and verapamil effectively inhibited electrically induced calcium transients. Next, C2C12 cells were exposed to these agents during conditions that induce myocyte differentiation. In the presence of nifedipine and verapamil, myoblasts failed to form myotubes. Dantrolene and thapsigargin, which decrease intracellular calcium by different mechanisms, also inhibited differentiation. In addition, nifedipine and verapamil inhibited the expression of myosin heavy chain and myogenin, two markers of skeletal myoblast differentiation. In contrast, levels of the transcriptional factor Myf5, which is expressed in undifferentiated myoblasts, did not decline. Calcium channel blockade also prevented the expression of a reporter driven by the skeletal muscle α-actin promoter. These data demonstrate that lowering intracellular calcium levels inhibits the differentiation of skeletal myoblasts into mature myotubes.

Mammalian myogenic differentiation is a complex process that involves maturation of myoblasts into mature myocytes (1, 2). These events lead to the activation of muscle-specific genes and the repression of genes required for cell proliferation, both of which are necessary for differentiation (1–3). This genetic program requires the action of a number of transcriptional factors and nuclear proteins (1–4). Our understanding of the signaling molecules that link extracellular signals to changes in transcriptional factor activity and subsequent gene expression is beginning to evolve.

Calcium is an intracellular messenger that is important in muscle cells for excitation-contraction coupling (reviewed in Refs. 3, 5). First, action potentials open voltage gated, L-type calcium channels to allow small quantities of calcium to enter the cell (so called “sparks”). This triggers calcium release from the sarcoplasmic reticulum (so called “calcium transients”). This larger wave of intracellular calcium leads to direct activation of the contractile apparatus, myosin cross-bridge cycling, and muscle contraction. Second, through the action of calcium/calcmodulin-dependent protein kinase II (CaMK II)1 and other kinases and phosphatases, calcium can indirectly alter the activity of proteins involved in contraction, calcium release, and calcium reuptake (3).

In addition to regulating cellular contraction, calcium acts as an intracellular messenger to modulate many aspects of cell physiology. Calcium-signaling pathways modulate the expression of muscle-specific genes during differentiation by altering the phosphorylation and activity of a number of transcriptional factors (reviewed in Ref. 3, 4). More specifically, calcium regulates the function of phosphatases and kinases (e.g. calcineurin, CaMK and protein kinase C) that can alter the localization and function of transcriptional activating factors such as cAMP response element-binding protein, MEF2, Myf5, MyoD, myogenin, nuclear factor of activated T-cells, and serum response factor (3, 4, 6–11).

Based on these observations, it is possible that intracellular calcium may play a role in the differentiation of striated myocytes by altering gene expression. To examine this issue, we studied the effects of decreased intracellular calcium levels in C2C12 cells, which are a favored model of skeletal muscle differentiation (12–14). Using three independent indices of myoblast differentiation, we now demonstrate that intracellular calcium levels play a crucial role in muscle cell maturation.

EXPERIMENTAL PROCEDURES

Materials—Medium and bovine serum were obtained from American Type Tissue Collection (Manassas, VA). All other culture supplements were from Invitrogen. Fluo-3 acetoxyethyl ester (Fluo-3 AM) and pluronic F-127 were from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma. Anti-myosin heavy chain antibody (MF-20) was developed by Donald Fischman and obtained from the Developmental Studies Hybridoma Bank, University of Iowa (15).

Cell Culture—C2C12 cells (ATCC no. CRL-1772) were grown on plastic or glass culture substrates as described (14). Briefly, cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 μM pyruvate, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate until 90–100% confluence. Cells were induced to differentiate using a low serum differentiation medium (Dulbecco’s modified Eagle’s medium, pyruvate, penicillin, streptomycin, and 2% horse serum). Upon induction of differentiation, medium containing vehicle or drugs was changed daily. Cells were cultured at 37 °C in a humidified incubator containing 5% CO2. Unless otherwise indicated, cells were harvested after 4 days in differentiation medium.

Calcium Imaging—Cells were labeled for 15 min with Fluo-3 AM diluted in differentiation medium to a final concentration of 5 μM from a stock solution of 885 μM Fluo-3 AM and 20 μM pluronic F-127 (per manufacturer’s instructions). Cells grown on cover slips were incubated

* This work was supported in part by National Institutes of Health Grant HL58442 (to S. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Pfizer Postdoctoral Fellow. To whom correspondence should be addressed: Dept. of Pediatrics, Div. of Cardiology, Yale University School of Medicine, 302 LCI, 333 Cedar St., P. O. Box 208064, New Haven, CT 06520-8064. Tel.: 203-785-2022; Fax: 203-737-2786; E-mail: george.porter@yale.edu.

|| Donaghue Medical Research Foundation Investigator.

1 The abbreviations used are: CaMK, calcium/calmodulin-dependent protein kinase; Fluo-3 AM, fluo-3 acetoxyethyl ester; ANOVA, analysis of variance.
Calcium Modulates Myocyte Differentiation

28943

in 1 ml of a solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl
to induce calcium release (calcium transients), samples were stimulated using a Grass SD6 stimulator (Quincy, MA). Platinum electrodes were placed 1.7 cm apart, and current flowed roughly parallel to the long axis of the myotube. Initial voltage response curves demonstrated that calcium transients occurred with a stimulation threshold of 15–30 V and a plateau of 50 V. Therefore, all further stimulations were performed at 30–40 V for 100 ms. Samples were stimulated, and images were recorded three times for each condition. Images were obtained 0.3 frames/s for a total of 5 s. The frame with the highest stimulated intensity is presented as a still image, and each image is linked to a QuickTime movie played at 3× speed. The corresponding movie files are: A, nifedipine 0 μM; B, nifedipine 1 μM; C, nifedipine 10 μM, and D, nifedipine 100 μM. Scale bar = 20 μM.

FIG. 2. Nifedipine and verapamil eliminate electrically stimulated calcium transients. C2C12 myotubes labeled with Fluo-3 AM were electrically stimulated, and fluorescence intensity was measured over time. A, representative example of fluorescence intensity of a C2C12 cell stimulated in the presence of increasing concentrations of nifedipine. Prior to any treatment (0 μM), stimulation caused a transient rise in intracellular calcium (●). Treatment with 0.1 (●), 1 (▲), 10 (●), and 100 (X) μM nifedipine decreased then abolished these calcium transients. B, peak fluorescence (±S.E.) during electrical stimulation in the presence of different concentrations of nifedipine or verapamil. Treatment with vehicle had no significant effects (not shown), whereas both nifedipine and verapamil caused a concentration-dependent decrease in the amplitude of the calcium transient. Means were compared by repeated measures using ANOVA with Dunnett’s post hoc testing (∗, p < 0.001; **, p < 0.05, significantly different from 0 μM of each agent).

Austin, TX). Probes were radiolabeled using the Prime-It RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA) and Redivue α-32PdCTP (3000 Ci/mmol, 10 μCi/ml, Amersham Bioscience) per the manufacturer’s instructions.

Luciferase Expression Assays—Reporter assays using a luciferase expression vector driven by the skeletal α-actin promoter (SK-Luc, provided by Dr. Robert Schwartz) were performed as described (14). Briefly, C2C12 cells were grown in 12-well plates to 50–75% confluence and were transfected with SK-Luc (1 μg/well) using LipofectAMINE 2000 (Invitrogen). To control for transfection efficiency, cells were co-transfected with pSV-β-galactosidase (35 ng/well, Promega, Madison, WI). When confluent, samples were then differentiated in the presence of vehicle or drug for 4 days as above. Samples were processed for luciferase and β-galactosidase expression using the luciferase and β-galactosidase assay systems with reporter lysis buffer (Promega, per the manufacturer’s instructions). Luciferase measurements were made using a luminometer (Turner Designs, Model TD-20/20, Sunnyvale, CA), and β-galactosidase was measured using a Bio-Rad Model 550 microplate reader.

Statistics—Data are presented as means and standard errors of the mean (S.E.). Except as noted, at least three separate experiments were performed.
performed for each condition. All quantifiable data were saved to Microsoft Excel or GraphPad Prism. Statistically significant differences between groups were evaluated using one-way or repeated measures ANOVA with Dunnett’s post hoc testing using GraphPad Prism (p < 0.05).

RESULTS

Calcium Channel Blockade Decreases C2C12 Cellular Calcium Levels—To begin to examine the role of calcium on myocyte differentiation we first examined the effects of L-type calcium channel blockade on intracellular calcium levels in C2C12 cells. Changes in intracellular calcium concentrations were assessed by confocal microscopy using the calcium-sensitive dye, Fluo-3 AM. Cultures were studied after 4 days in differentiation medium.

To define patterns of Fluo-3 response under normal conditions, cells were stimulated at different voltages (5–50 V) to generate voltage-response curves. In all cases the threshold for a response in Fluo-3 signal was 15–30 V. At 50 V, we saw maximal responses in all cells. Thus, in all subsequent studies cells were stimulated at 30–40 V.

Next, to determine the effects of drugs on calcium release, samples were examined in the presence or absence of increasing concentrations of nifedipine and verapamil, two antagonists of L-type calcium channels. Both agents decreased the peak calcium response to electrical stimulation in a concentration-dependent manner, although nifedipine was more effective at the concentrations used (Figs. 1 and 2). Control experiments using vehicle demonstrated that the effects were not due to nonspecific effects of the experimental protocol over time (not shown).

Decreasing Intracellular Calcium Levels Inhibits Morphologic Differentiation of C2C12 Myoblasts—Having demonstrated that nifedipine and verapamil decrease intracellular calcium levels, we next examined the effects of reduced intracellular calcium concentrations on myocyte differentiation. In the presence of differentiation medium, C2C12 cells mature into multinucleated myotubes with a functional contractile apparatus (12, 20). Therefore, confluent cultures of C2C12 cells were incubated in differentiation medium in the presence of vehicle, nifedipine, or verapamil for 4 days. The morphology of each specimen was first assessed using phase microscopy. Cul-
Cultures to differentiate (Fig. 3), either nifedipine or verapamil inhibited the ability of these agents known to decrease intracellular calcium by mechanisms due to decreased calcium levels, we next treated C2C12 cells with 7.6% of control values, respectively.

Positive myotubes (Fig. 3) myoblasts and numerous multinucleated, myosin heavy chain—impressions were confirmed. Control experiments demonstrated that nifedipine and verapamil inhibiting the expression of structural genes that are normally up-regulated during myocyte differentiation in a concentration-dependent manner (Figs. 3 and 4).

Calcium Channel Blockade Alters the Expression of Muscle-Specific Transcriptional-Activating Factors—After observing that nifedipine and verapamil inhibited cellular differentiation, we next examined the effects of these agents on the expression of two muscle-specific transcriptional activating factors, myogenin and Myf5. During myoblast differentiation the expression of myogenin is increased, while that of Myf5 is decreased (1, 2, 22). To address this issue, Northern blotting experiments were performed using RNA from C2C12 cells treated with nifedipine or verapamil during differentiation. Myogenin and Myf5 levels were quantified using a phospho-imaging device.

As expected, we observed that myogenin gene expression increased during differentiation (not shown) (1, 2, 22). However, cultures treated with nifedipine or verapamil maintained high levels of Myf5 expression, although the levels of Myf5 expression in cell treated with verapamil were not statistically significant from control (Fig. 5, A and B). As with myogenin, the effects of these agents on Myf5 expression were concentration-dependent (Fig. 5C).

Skeletal α-Actin Expression Is Inhibited by Calcium Channel Blockade—The previous experiments showed that calcium channel blockade inhibits morphologic changes and alters transcriptional factor expression during myoblast differentiation. Next, to assess if decreased intracellular calcium levels alter the expression of structural genes that are normally up-regulated during myocyte differentiation, the effects of nifedipine and verapamil on α-actin gene expression were examined.

Cultures of C2C12 cells were transfected with SK-Luc, a Calcium channel blockade decreases reporter expression. C2C12 cells were transfected with SK-Luc, a luciferase reporter vector driven by the skeletal α-actin promoter, and differentiated in the presence of vehicle, nifedipine, or verapamil. Relative luciferase activity (normalized to β-galactosidase activity) for each treatment (± S.E.) is presented as a percent of the vehicle control. Each column is the mean of at least three separate experiments in which three replicates were performed. Data were analyzed for significance using ANOVA with Dunnett’s post hoc testing (*, p < 0.001).

Calcium channel blockade decreases myogenin and increases Myf5 expression. Total RNA, extracted from C2C12 cells incubated in differentiation medium with vehicle or various concentrations of nifedipine or verapamil, was transferred to a nylon membrane for northern blotting. A. example of a Northern blot labeled for myogenin, Myf5, and 18 S rRNA (to demonstrate equal loading of RNA). Myogenin (B) and Myf5 (C) labeling on Northern lots were quantified and normalized to control. Each column represents at least four different experiments. Data were analyzed for significance using ANOVA with Dunnett’s post hoc testing (*, p < 0.05).

Vehicle-treated cultures contained few undifferentiated myoblasts and numerous multinucleated, myosin heavy chain—positive myotubes (Fig. 3, A and F). In contrast, treatment with either nifedipine or verapamil inhibited the ability of these cultures to differentiate (Fig. 3, B–E, G–J). Calcium channel blockade decreased both the number and the size of differentiated myotubes. These effects were concentration-dependent, and the effective concentrations correlated with those required to attenuate electrically stimulated calcium transients (Figs. 1 and 2).

When the effects of nifedipine and verapamil on differentiation were quantified using a differentiation index, these visual impressions were confirmed. Control experiments demonstrated the emergence of multinucleated myotubes during incubation in differentiation medium with 67% of the nuclei present in differentiated cells after 4 days (Fig. 4A). In contrast, both nifedipine and verapamil significantly decreased the ability of C2C12 cells to differentiate (Fig. 4B). This effect was concentration-dependent, with 100 µM nifedipine and 100 µM verapamil reducing the differentiation index to 25.1% and 7.6% of control values, respectively.

To further demonstrate that these effects on differentiation are due to decreased calcium levels, we next treated C2C12 cells with agents known to decrease intracellular calcium by mechanisms other than calcium channel blockade. Differentiating C2C12 cells were treated with dantrolene, which inhibits calcium release from the sarcoplasmic reticulum, or thapsigargin, which inhibits calcium reuptake by the sarcoplasmic reticulum and eventually depletes total cellular calcium (21). The doses used are known to effectively decrease intracellular calcium levels and to alter gene expression in skeletal myotubes (21). Treatment with either agent led to a qualitative and quantitative inhibition of myocyte differentiation in a concentration-dependent manner (Figs. 3 and 4).
luciferase expression vector driven by the skeletal α-actin promoter that has been validated in previous studies of C2C12 cell and primary myocyte differentiation (14, 22, 23). Non-confluent C2C12 cultures were transfected with SK-Luc and were allowed to differentiate in medium containing vehicle, nifedipine, or verapamil. After 4 days in differentiation medium, specimens were assayed for luciferase expression.

As expected from previous experiments, specimens treated with vehicle expressed high amounts of luciferase (14, 22, 23). However, in a concentration-dependent manner, both nifedipine and verapamil significantly inhibited luciferase expression (Fig. 6). Normalized luciferase levels for cultures treated with nifedipine were 80.4, 81.8, 37.4, and 7.7% at 1, 10, 50, and 100 μM, respectively. Specimens treated with 1, 10, 50, and 100 μM verapamil expressed luciferase at 92.1, 94.5, 25.6, and 0.6% of control, respectively. Therefore, this third index demonstrates that lowering intracellular calcium levels inhibits C2C12 differentiation.

**DISCUSSION**

It is becoming clear that many extracellular signals regulate the expression of genes essential for striated myocyte differentiation (7, 10, 11, 19, 24). However, the intracellular pathways that transduce these signals to the nucleus are only beginning to be understood. Previous reports have suggested that calcium-activated signaling cascades may play an important role in intracellular signaling during myocyte differentiation (7, 11, 13, 25, 26). Using C2C12 cells as a model of myocyte differentiation, we demonstrate that the maintenance of normal intracellular calcium concentrations is vital to myocyte gene expression and differentiation.

Using a calcium-sensitive dye and confocal microscopy, we first demonstrated that electrically stimulated calcium transients were inhibited by calcium channel blockade. These results are not unexpected because differentiating C2C12 cells are known to express L-type calcium channels (27). These data also agree with previous reports that calcium channel blockade decreases the frequency and amplitude of calcium transients in C2C12 cultures at normal intracellular calcium levels and in primary cultures of skeletal myocytes (25, 28–30). It is interesting that verapamil was not as effective as nifedipine in these experiments. The binding of verapamil to L-type calcium channels is enhanced if the channel is actively opening and closing (31). Therefore, these findings were not unexpected due to the acute treatments used in these experiments. In addition, other experiments demonstrated that verapamil lowered calcium transient amplitude more effectively if specimens were stimulated at 0.5 Hz during each 2-min treatment period.2

Having established the concentrations of nifedipine and verapamil required to effectively decrease intracellular calcium concentration, we found that channel blockade profoundly inhibited myoblast differentiation. Visual observations of C2C12 cells induced to differentiate in the presence of nifedipine or verapamil demonstrated that decreasing intracellular calcium levels inhibited the formation of multinucleated myotubes. Additional quantitative indices supported this finding. First, immunolabeling and calculation of a differentiation index quantified gross morphologic observations. Second, decreased expression of myogenin and increased expression of Myf5 in cells treated with both drugs demonstrated that the genetic program required for normal differentiation was disrupted. Third, the expression of actin and myosin, two structural genes present in the mature contractile apparatus, was inhibited by calcium channel blockade.

We then treated C2C12 cells with dantrolene and thapsigargin to demonstrate that these effects were due to decreased intracellular calcium levels and not due to some other effect of calcium channel blockade. Dantrolene inhibits calcium release from the sarcoplasmic reticulum and thus decreases sarcoplasmic calcium (21). In contrast, thapsigargin blocks reuptake of calcium into the sarcoplasmic reticulum and thus eventually depletes total cellular calcium (21). As with nifedipine and verapamil, treatment of C2C12 cells with these agents also prevented morphologic differentiation. Therefore, these data support the hypothesis that decreased intracellular calcium levels inhibit differentiation of muscle cells.

Potential mechanisms of this calcium-mediated modulation of differentiation and gene expression include alterations in protein degradation, in mRNA stabilization, or in gene transcription. The latter mechanism appears to be most important during differentiation, but evidence exists to support a role for the former two mechanisms. For example, calpain-mediated protein degradation appears to allow cell fusion and differentiation of C2C12 cells (13). In addition, increased levels of intracellular calcium appear to stabilize mRNA encoding the inward-rectifying potassium channel 1 (26).

However, regulation of gene transcription appears to play a major role in calcium-mediated modulation of differentiation. Recent reports suggest that this occurs via the activation of a number of phosphatases and kinases, including calcineurin, CaMK isofoms, and protein kinase C. These enzymes alter the activity of transcriptional activating factors, such as CAMP-response element-binding protein, MEF2, Myf5, myogenin, nuclear factor of activated T cells, and serum response factor (3, 4, 6–11). Our results provide a basis for further investigation of these potential mechanisms.

In conclusion, our data suggest that mammalian skeletal myoblast differentiation is dependent upon normal intracellular calcium concentrations. Future experiments using other methods to alter intracellular calcium levels should further define the relationship between intracellular calcium concentrations and myoblast differentiation. In addition, these experiments may also help determine which calcium-dependent pathways contribute to the integration of signals that drive skeletal myofiber development.

**Acknowledgments**—We thank Drs. Lei Wei and Robert Schwartz for the use of SK-Luc. We also thank Thomas O’Reilly-Pol for technical expertise.

**REFERENCES**

1. Molkentin, J. D., and Olson, E. N. (1996) *Curr. Opin. Genet. Dev.* 6, 445–453
2. Sabourin, L. A., and Rudnicki, M. A. (2000) *Clin. Genet.* 57, 16–25
3. Frey, N., McKinsey, T. A., and Olson, E. N. (2000) *Nat. Med.* 6, 1221–1227
4. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) *Curr. Opin. Genet. Dev.* 11, 497–504
5. Ballé, C. W., and Shorofsky, S. R. (1998) *Cardiovasc. Res.* 37, 290–299
6. Bito, H. (1998) *Cell Calcium* 23, 143–150
7. Delling, U., Tureckova, J., Lim, H. W., De Winde, L. J., Rotwein, P., and Molkentin, J. D. (2000) *Mol. Cell. Biol.* 20, 6609–6611
8. Hardingham, G. E., Cruzado, P. F., Chawla, S., and Bading, H. (1998) *Cell Calcium* 23, 131–134
9. Hermann, S., Snaar-Kocktett, J., Onions, J., Hughes, K., and Grundstrom, T. (1998) *Cell Calcium* 23, 135–142
10. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. (1992) *Cell Calcium* 11, 1181–1194
11. Simansari, C., Wu, M. J., Xu, Y. K., Marcincio, T., Yeoh, T., Allen, D. G., Harvey, R. P., and Graham, R. M. (1999) *Nature* 400, 576–581
12. Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C., and Webster, C. (1985) *Science* 230, 758–766
13. Tsumura-Grove, C. J., Wirt, D., Thompson, V. F., Allen, R. E., and Goll, D. E. (1999) *Exp. Cell Res.* 247, 293–303
14. Wei, L., Zhou, W., Croissant, J. D., Johansen, F. E., Prywe, R., Balsabramanyam, A., and Schwartz, R. J. (1996) *J. Biol. Chem.* 271, 30287–30294
15. Bader, D., Masaki, T., and Fischman, D. A. (1982) *J. Cell Biol.* 95, 763–770
16. Lorenzon, P., Grohovaz, F., and Ruzzier, F. (2000) *J. Physiol.* 523, 499–507
17. Porter, G. A., Dmytryrenko, G. M., Winkelmann, J. C., and Bloch, R. J. (1992) *J. Cell Biol.* 117, 997–1005
18. Porter, G. A., Scher, M. G., Resneck, W. G., Porter, N. C., Fowler, V. M., and Bloch, R. J. (1997) *Cell Motil. Cytoskeleton* 37, 7–19
19. Ogilvie, M., Yu, X., Nicolas-Metral, V., Pulido, S. M., Liu, C., Ruegg, U. T., and
Calcium Modulates Myocyte Differentiation

Noguchi, C. T. (2000) *J. Biol. Chem.* **275**, 39754–39761
20. Kubo, Y. (1991) *J. Physiol.* **442**, 743–759
21. Vali, S., Carlsen, R., Pessah, I., and Gorin, F. (2000) *J. Cell. Physiol.* **185**, 184–199
22. Shimokawa, T., Kato, M., Ezaki, O., and Hashimoto, S. (1998) *Biochem. Biophys. Res. Commun.* **246**, 287–292
23. MacLellan, W. R., Lee, T. C., Schwartz, R. J., and Schneider, M. D. (1994) *J. Biol. Chem.* **269**, 16754–16760
24. Li, Y. P., and Schwartz, R. J. (2001) *FASEB J.* **15**, 1413–1415
25. Huang, C. F., Flucher, B. E., Schmidt, M. M., Stroud, S. K., and Schmidt, J. (1994) *Neuron* **13**, 167–177
26. Shin, K. S., Park, J. Y., Kwen, H., Chang, C. H., and Kang, M. S. (1997) *J. Biol. Chem.* **272**, 21227–21232
27. Varadi, G., Orłowski, J., and Schwartz, A. (1989) FEBS Lett. **250**, 515–518
28. Challet, C., Maechler, P., Wollheim, C. B., and Ruegg, U. T. (2001) *J. Biol. Chem.* **276**, 3791–3797
29. Lorenzon, P., Giovannelli, A., Ragozzino, D., Eusebi, F., and Ruzzier, F. (1997) *Eur. J. Neurosci.* **9**, 800–808
30. Bruton, J. D., Katz, A., and Westerblad, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3281–3286
31. Hofmann, F., Lacinova, L., and Klugbauer, N. (1999) *Rev. Physiol. Biochem. Pharmacol.* **139**, 33–87