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Reversible Block of the Calcium Release Channel/Ryanodine Receptor by Protamine, a Heparin Antidote

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Channel activity of the calcium release channel from skeletal muscle, ryanodine receptor type 1, was measured in the presence and absence of protamine sulfate on the cytoplasmic side of the channel. Single-channel activity was measured after incorporating channels into planar lipid bilayers. Optimally and suboptimally calcium-activated calcium release channels were inactivated by the application of protamine to the cytoplasmic side of the channel. Recovery of channel activity was not observed while protamine was present. The addition of protamine bound to agarose beads did not change channel activity, implying that the mechanism of action involves an interaction with the ryanodine receptor rather than changes in the bulk calcium concentration of the medium. The block of channel activity by protamine could be reversed either by removal by perfusion with buffer or by the addition of heparin to the cytoplasmic side of the channel. Microinjection of protamine into differentiated C_2C_{12} mouse muscle cells prevented caffeine-induced intracellular calcium release. The results suggest that protamine acts on the ryanodine receptor in a similar but opposite manner from heparin and that protamine can be used as a potent, reversible inhibitor of ryanodine receptor activity.

INTRODUCTION

Ryanodine receptors (calcium-induced calcium release channels; RyR) play a crucial role in most cell types, including muscle cells, neurons, and epithelial cells. They mediate the release of calcium ions from the endoplasmic/sarcoplasmic reticulum into the cytosol and thereby convert a number of extracellular stimuli into intracellular calcium signals. RyRs are large tetrameric proteins that show sequence similarity with inositol 1,4,5-trisphosphate (IP_3)-gated calcium channels of the endoplasmic/sarcoplasmic reticulum, but they are distinct in their biophysical and pharmacological properties (Smith *et al.*, 1986; Ehrlich and Watras, 1988; Supattapone *et al.*, 1988; Mignery *et al.*, 1989; Palade *et al.*, 1989, Ehrlich *et al.*, 1994). For example, highly negatively charged polyanions such as pentosan polysulfate, polyvinyl sulfate, and heparin increased the activity of RyRs, whereas they decrease the activity of IP_3 receptors (Bezprozvanny *et al.*, 1993). The authors of these studies reported that to get an increase in channel open probability, several polyanion molecules needed to bind to the RyR. They provided evidence for a mechanism in which the binding of polyanions to the RyR would increase the local negative charge of the receptor complex and thus attract more calcium ions to activate the receptor. Additional support for this mechanism is found in the facts that the endogenous RyR has a fixed negative surface charge and that this surface charge potentiates conduction and conveys divalent cation selectivity of the channel (Tu *et al.*, 1994).

In the present study, we analyzed the effects of protamine (clupeine) on the RyR. Protamines are a known antidote to heparin (Byun *et al.*, 1999), are highly positively charged, and are rich in basic amino acids (Felix, 1960). These proteins are known to bind tightly to DNA and are used in DNA-binding assays (Raukas and Mikelsaar, 1999). Several studies discuss the role of changes in the local charge of the RyR and the IP_3 receptor (IP_3R) after the addition of negatively charged compounds as a means to modulate channel activity (Ghosh *et al.*, 1988; Bezprozvanny *et al.*, 1993). In the present paper, we chose a similar approach, but we investigated the effect of adding a positively charged ligand (protamine) to modulate the activity of the RyR. The results complement the current view of the role of fixed charges in the regulation of the RyR and provide a tool to reversibly inactivate RyR function in physiological systems.

MATERIALS AND METHODS

All experiments described in this study were carried out in accordance with the appropriate National Institutes of Health and Yale University guidelines.

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Bilayer Experiments

Vesicles prepared from rabbit skeletal muscle sarcoplasmic reticulum were isolated in the presence of protease inhibitors by differential centrifugation (Kim *et al.*, 1983). Vesicles were divided into aliquots and stored at −80°C until needed for experiments. RyRs present in the sarcoplasmic reticulum vesicles were incorporated into planar lipid bilayers (Ehrlich and Watras, 1988) containing phosphatidylethanolamine and phosphatidylserine (3:1 [wt/wt]; Avanti Polar Lipids, Alabaster, AL) dissolved in decane (40 mg lipid/ml). A KCl gradient with the higher KCl concentration on the side of vesicle incorporation (*cis* side) was used to facilitate fusion. The experiments were performed with a 250 mM HEPES-Tris solution, pH 7.35, on the *cis* side and a 250 mM HEPES, 55 mM $Ba(OH)_2$ solution, pH 7.35, on the *trans* side of the bilayer. Histone-free protamine sulfate from herring sperm, protamine cross-linked to 4% beaded agarose, heparin (approximate average M_r, 6000), and all other reagents were obtained from Sigma Chemical (St. Louis, MO). Protamine cross-linked agarose was washed three times in buffer before its experimental use to avoid impurities of dissociated free protamine molecules in the preparation. Capacitance, ion permeability, and stability of the lipid bilayers were checked before, during, and after drug application and after removal of drugs from the system to exclude effects of the applied compounds on the phospholipids forming the lipid bilayer. We observed no changes in these bilayer properties. Experiments were recorded under voltage-clamp conditions with a holding potential of 0 mV. Data were filtered at 1 kHz and digitized at 3 kHz, directly transferred to a computer, and analyzed with pClamp version 6.0.3 (Axon Instruments, Burlingame, CA). The concentration of free calcium was adjusted as described by Fabiato (1988). If channels could not be activated by the addition of 1 μM calcium to the *cis* side, these channels were regarded as improperly inserted into the artificial bilayer and were not analyzed. However, in the majority of experiments, the addition of calcium indicated that the vesicles had fused with the bilayer such that the cytosolic part of the RyR faced the *cis* side. The data shown were obtained from four or more independent trials for each experiment.

Optical Recordings of Intracellular Calcium Concentrations

C_2C_{12} cells (American Type Culture Collection [Rockville, MD] CRL 1772) were cultured and differentiated as described previously (Bennett *et al.*, 1996; addition of 1% horse serum and 0.5% insulin transferrin selenite to the growth medium). The expression of RyRs after differentiation of C_2C_{12} cells was monitored with standard Western blotting techniques (Koulen *et al.*, 1997; primary antibody, MA3-916 from Affinity Bioreagents [Golden, CO]; peroxidase detection, LumiGLO substrate kit from Kirkegaard & Perry Laboratories [Gaithersburg, MD]). Cells expressed RyRs after differentiation, as indicated by the high-molecular-mass band in Figure 1 of ~500–600 kDa corresponding to the calculated molecular mass of the RyR (Marks *et al.*, 1989; Takeshima *et al.*, 1989). Cell were grown on glass coverslips to a subconfluent density for calcium imaging. During the experiments, the cells were kept in extracellular solution (ECS; in mM: NaCl, 137; KCl, 5; $CaCl_2$, 2; Na_2HPO_4, 1; $MgSO_4$, 1; HEPES, 10; glucose, 22; pH 7.4). Cells were incubated in ECS containing 4 μM cell-permeant fluo-3 (fluo-3 acetoxymethyl ester, Molecular Probes, Eugene, OR) with 0.05% DMSO for 15–30 min and were washed in ECS before the optical recording. The fluo-3 fluorescence present in loaded cells was measured with a Bio-Rad (Richmond, CA) MRC-1024 system equipped with a Zeiss (Thornwood, NY) Axiovert S100 microscope. While the dye-loaded cells were excited with light of 488 mm wavelength, increases in intracellular calcium were measured at the emission wavelength maximum of fluo-3 (522 nm). Fluo-3, upon binding of calcium ions, undergoes a 40- to 200-fold increase in fluorescence (Harkins *et al.*, 1993). Changes in fluorescence intensity were calculated by dividing the measured fluorescence intensity during drug application (F) by the measured average baseline fluorescence intensity (F/F_0). Images were acquired every 500 ms with the photomultiplier tubes of the Bio-Rad MRC-1024 system. Nonstimulus-related, spontaneously occurring changes in fluorescence intensity, as well as changes after the application of control substances, were in the range of 1–5% of F/F_0. The present data provide a qualitative estimate of the drug-evoked calcium responses in C_2C_{12} cells, because the F/F_0 values do not correlate linearly to changes in the intracellular calcium concentration. During the experiments, cells were kept in a perfusion chamber on the microscope stage at room temperature and were superfused continuously with ECS at a flow rate of 1 ml/min. Cells were injected with 10–100 fl of protamine sulfate (4 mM) or water as a control with the use of a micromanipulation and microinjection setup (Eppendorf microinjector 5242, Eppendorf micromanipulator 5171) and borosilicate micropipettes (Eppendorf-Netheler-Hinz, Hamburg, Germany). To monitor injected cells, TRITC-dextran was injected together with either protamine or control substances. After injection, cells were kept in ECS for 30–60 min before bath-application of drugs took place. The viability of injected cells was assessed with a viability/cytotoxicity kit for animal cells (Molecular Probes). This test specifically stains intact live cells and dead cells with damaged cellular membranes with the use of cell-permeant and cell-impermeant fluorescent dyes. Only 2–5% of the injected cells were not viable. Such cells could be recognized easily with bright-field microscopy as apoptotic and were not included in the experiments. Thapsigargin (1–10 μM; Calbiochem-Novabiochem, San Diego, CA) was applied to protamine-injected, control-injected (water, buffer, or impalement alone without injection of substances), and noninjected control cells. Thapsigargin released calcium from intracellular stores irrespective of pretreatment. Caffeine (5–50 mM) or an identical amount of ECS or water was bath-applied directly into the chamber, which had a volume of 1 ml. The responses shown in the present study were obtained from four independent trials for each experimental condition.

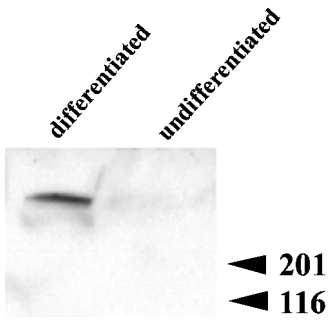
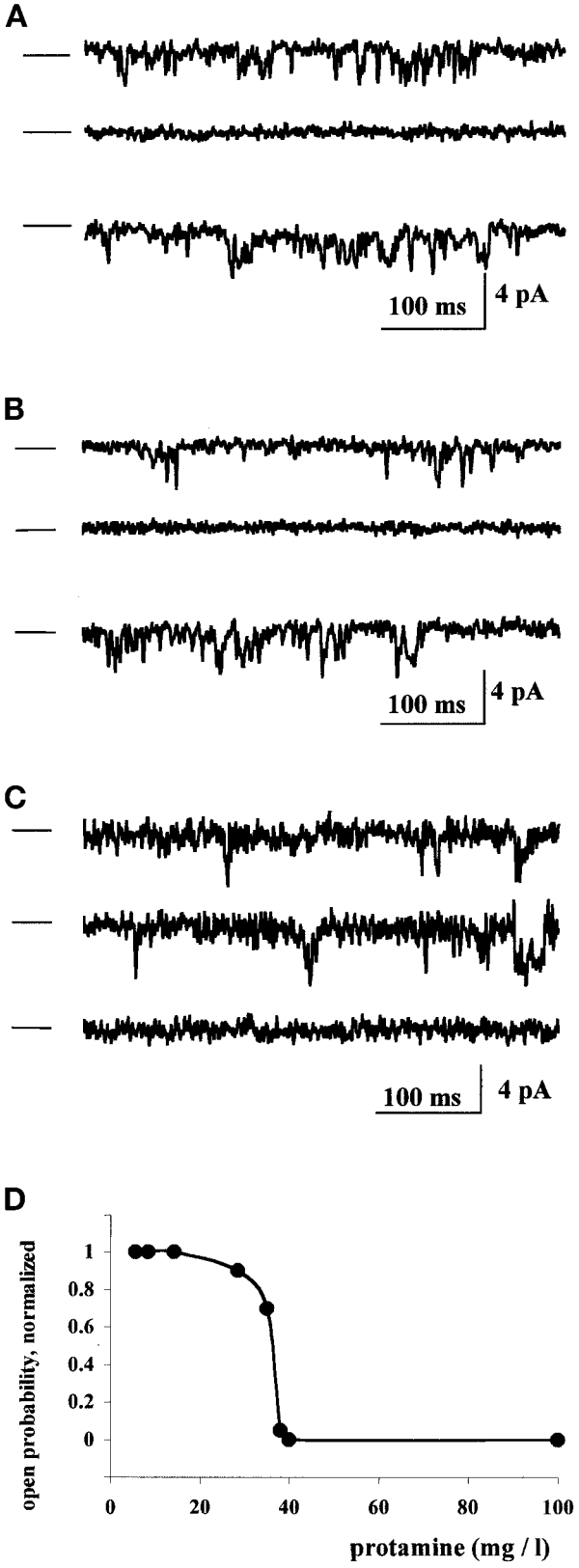


Figure 1. Western blot probing for the expression of RyR by undifferentiated and differentiated C_2C_{12} cells. C_2C_{12} cells were lysed, and their proteins were denatured in sample buffer. Protein (60 μg) was loaded and separated by SDS-PAGE in a 4–12% polyacrylamide gradient gel. The primary antibody (MA3-916) directed against the RyR detected a high-molecular-mass band of ~500–600 kDa with the use of peroxidase detection and a LumiGLO substrate kit (KPL) in differentiated cells, confirming the findings of Bennett and colleagues (1996). RyR signals could not be detected in undifferentiated cells because expression was below the detection level. Comparison of the RyR signals in undifferentiated and differentiated C_2C_{12} cells clearly shows the strong increase in RyR after differentiation of C_2C_{12} cells. Numbers indicate the positions and molecular masses of protein standards in kDa.

RESULTS

In the present study, we combined single-channel electrophysiological recordings of RyRs with optical imaging of



intracellular calcium transients in intact cells expressing RyRs at high levels (Figure 1) (Bennett *et al.*, 1996). These two assay systems were used to study and evaluate the effects of protamine on RyRs.

Protamine Blocks RyR Activity Reversibly

RyR1 from rabbit skeletal muscle was incorporated into bilayers (Figures 2 and 3), and the biophysical properties were compared with results reported in previous publications (Quinn *et al.*, 1998). The single-channel conductance was 115 ± 6 pS with barium as the current carrier, the bell-shaped calcium-response curve was identical to that in experiments reported previously (Quinn *et al.*, 1998) (Figure 3, squares), and the channel activity was blocked by ruthenium red. The single-channel open probability was typically in the range of 25–45% at maximally activating calcium concentrations (pCa 5.8). Data from individual experiments were normalized before they were averaged because activity levels vary among individual channels. Channel activity of the ryanodine receptor was measured in the presence and absence of protamine added to the cytoplasmic side of the channel. Protamine was added after channels were activated with calcium (Figure 2, A and B, middle trace in each panel).

When the protamine-containing buffer at the cytoplasmic side of the channel was removed by perfusion with protamine-free buffer, the inhibition of the channel activity could be reversed (Figure 2A, bottom trace). Block of the channel activity by protamine could also be overcome by the addition of 10–40 μg/ml heparin to the cytoplasmic side of the channel (Figure 2B, bottom trace). After the addition of heparin, the channel open probability was typically higher than the baseline activity before the addition of protamine, presumably as a result of the effect of excess heparin on the RyR (Bezprozvanny *et al.*, 1993). When the RyR was blocked with protamine, no recovery of activity or desensitization to

Figure 2. RyR1 from rabbit skeletal muscle sarcoplasmic reticulum vesicles is inhibited by protamine sulfate (clupeine). Single-channel activity was recorded in the presence of 0.3 μM free calcium on the cytoplasmic side of the RyR. Zero current levels are indicated by the bar at the left of each trace, and openings are shown as downward deflections. Panels A, B, and C show recordings from three different experiments. The top trace of each panel shows channel activity before drug application. In A and B, the addition of 40 μg/ml protamine to the cytosolic side of the channel leads to inactivation of the RyR and complete loss of channel activity (middle trace). Removal of protamine by perfusion with buffer (A) or by the addition of 40 μg/ml heparin to the cytoplasmic side of the channel (B) restores channel activity (bottom trace). The addition of protamine cross-linked with beaded agarose (activity equivalent to 40 μg/ml protamine) to an active RyR channel does not change the open probability (C, middle trace); further addition of free protamine (40 μg/ml), however, blocks channel activity (C, bottom trace). In D, the effects of different protamine concentrations on RyR channel activity in the presence of 0.3 μM free calcium on the cytoplasmic side of the RyR were tested in three independent experiments. Protamine concentrations of <15 μg/ml did not affect active RyR channels, whereas 20–35 μg/ml protamine reduced channel open probability slightly, and concentrations of 38 μg/ml or greater inhibited channel activity substantially. A protamine concentration of >40 μg/ml completely inhibited RyRs. Protamine addition never increased channel activity. Half-maximal inhibition was obtained at 37 ± 1 μg/ml (n = 3), and a Hill coefficient >4 was observed.

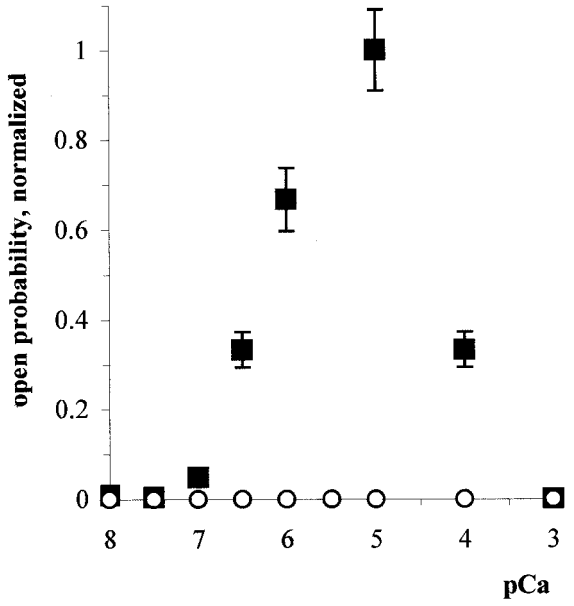


Figure 3. Calcium dependence of the normalized open probability of RyR channels in the absence and presence of protamine. Channel activity was recorded in the presence of different calcium concentrations on the cytosolic side of the RyR. Individual points represent means ± SD for control measurements (n = 6; ■) and for the open probability in the presence of 40 μg/ml protamine (n = 3; ○). If vertical error bars are not visible, the SD of the open probability is smaller than the symbol.

the compound was observed over time. On average, the absence of the channel activity in the presence of protamine was recorded for 5 min before further manipulations were carried out. In single experiments, periods of channel block up to 20 min were recorded. In each case, channel activity could be regained only by removing protamine or adding heparin (Figure 2, A and B, bottom traces).

Biophysical Characteristics of the Protamine-RyR Interaction: Direct Action of Protamine

To test a possible interaction of protamine sulfate with calcium ions and to investigate the nature of the interaction of protamine with the RyR, additional experiments were carried out. Protamine cross-linked to agarose was added to the cytoplasmic side of the channel. RyR channel activity was not altered after the addition of beaded protamine independent of the concentration of protamine agarose (Figure 2C, middle trace). Concentrations of cross-linked protamine equivalent to and higher than the concentration of free protamine (40 μg/ml) used to inhibit channel activity (Figure 2, A and B) were tested. The concentration of cross-linked protamine was estimated based on the binding capacity for defined DNA concentrations. When protamine-agarose was added to the cytosolic side, activity was maintained (Figure 2C, middle trace), and subsequent addition of free protamine blocked calcium release channel activity (Figure 2C, bottom trace). The effects of different protamine concentrations on RyR channel activity were tested in three independent experiments. Protamine concentrations of <15 μg/ml had no effect on active RyR channels; concentrations between 20 and 35 μg/ml reduced channel open probability slightly; and concentrations of 38 μg/ml or greater inhibited channel activity substantially. A protamine concentration of >40 μg/ml completely inhibited RyRs (Figure 2D; half-maximal inhibition at 37 ± 1 μg/ml, n = 3). A Hill coefficient of >4 in a three-parameter Hill equation [$f = (a \times x^b)/(c^b + x^b)$] fit indicates that more than four molecules of protamine must bind to the RyR to induce block.

The effect of protamine sulfate on the RyR was tested over the range of calcium concentrations relevant for RyR channel activation (Figure 3). At every calcium concentration tested from 0.01 μM to 1 mM, protamine blocked channel activity. Even when the calcium concentration produced very low activity of the RyR channel (pCa 8–7.5, pCa 3), protamine blocked this activity. Activation of RyRs by protamine was never observed.

Protamine Blocks RyR Channel Activity in Intact Cells

To relate the effects of protamine on the single-channel properties of RyRs to functions in living cells, calcium imaging experiments were performed in differentiated C_2C_{12} mouse muscle cells. Compared with undifferentiated C_2C_{12} cells, differentiated mouse C_2C_{12} cells express high levels of RyRs (Bennett *et al.*, 1996) (see MATERIALS AND METHODS and Figure 1). Once differentiation of C_2C_{12} cells with strong RyR expression is induced (Bennett *et al.*, 1996), these cells show a distinct cytosolic calcium transient mediated by ryanodine-sensitive intracellular calcium stores upon stimulation with caffeine (Figure 4, A–C) (Lorenzon *et al.*, 1997). In Figure 4, A–C, the cell indicated by the arrowhead was injected with protamine, whereas the cell labeled with the arrow was not injected. The control cell shows a change in fluorescence that resolves with time, whereas the injected cell does not. Panels B and C show the calcium response 16 and 38 s, respectively, after caffeine application. The time course of caffeine-induced calcium changes is shown in Figure 4, D and E. Microinjection of protamine into differentiated C_2C_{12} cells prevented caffeine-induced intracellular calcium release (Figure 4E). If the cells were injected with equal amounts of buffer or water, caffeine-induced responses as seen in noninjected cells were observed (Figure 4D). To monitor potential artifacts introduced by the microinjection of substances into differentiated C_2C_{12} cells, several control experiments were performed. The microinjection of protamine did not interfere with the viability of cells and left the intracellular calcium stores intact. Compared with cells that were impaled with the injection needle without any substance being injected or with buffer-injected cells, cells that were injected with protamine showed no altered viability. The viability of injected cells was assessed with a viability/cytotoxicity kit for animal cells (Molecular Probes). The integrity of intracellular calcium stores of protamine-injected compared with control-injected cells was determined by stimulating the release of calcium from intracellular stores. Thapsigargin, a cell-permeable inhibitor of the endoplasmic reticular calcium-ATPase, was applied to protamine-injected, control-injected, and noninjected control cells, and calcium was released from intracellular stores irrespective of pretreatment. The thapsigargin response of a protamine-

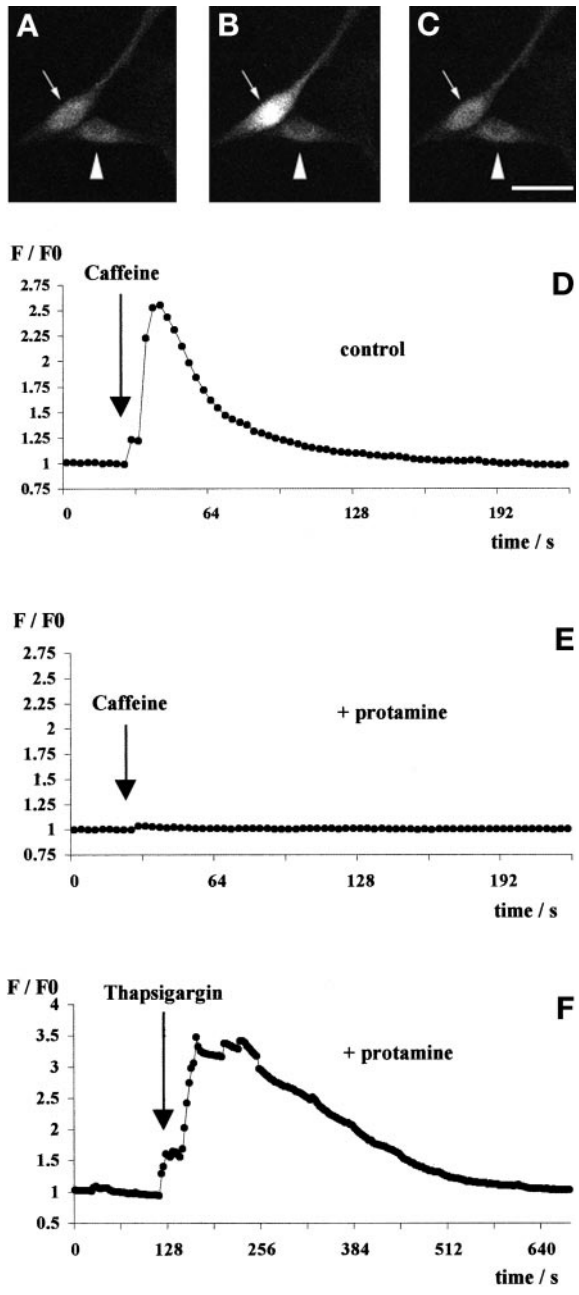


Figure 4. Optical recordings of intracellular calcium concentrations in differentiated C_2C_{12} cells. Cells were loaded with fluo-3 and stimulated with caffeine, and changes in the emission wavelength maximum of fluo-3 attributable to changes in intracellular calcium concentrations were monitored. A–C show the responses of two C_2C_{12} cells to a 20 mM caffeine stimulus. Increases in brightness represent increases in intracellular calcium concentration. (A) Baseline fluorescence of a cell injected with 50 fl of 4 mM protamine sulfate (arrowhead) and a control cell (arrow). (B) The same cells 16 s after the addition of caffeine. The intracellular calcium concentration in the control cell (arrow) increases significantly, whereas the protamine-injected cell (arrowhead) displays no significant calcium transients. (C) The cells after 38 s, with the calcium concentration of the control cell (arrow) returned to baseline levels. Scale bar in C for A–C, 50 μm. D and E show responses of two cells representative of injected cell is shown in Figure 4F, revealing intact intracellular calcium stores.

DISCUSSION

In the present study, protamine was found to be a potent inhibitor of RyR calcium channel function in single-channel recordings (Figures 2 and 3) as well as in optical recordings of calcium concentration changes in living cells (Figure 4). Protamines are widely used heparin antidotes (Byun *et al.*, 1999), are highly positively charged molecules (Felix, 1960), and are mixtures of closely related basic proteins. In the present study, clupeine was chosen because its composition is well characterized and its three components (YI, YII, and Z) are fully sequenced. The M_r of YI is 4841, that of YII is 4777, and that of Z is 4163 (all in the hydrochloride form; Iwai *et al.*, 1971; Suzuki and Ando, 1972a,b).

The effects of heparin and other highly negatively charged molecules on the RyR are well described and consist of an increase in channel open probability (Ghosh *et al.*, 1988; Bezprozvanny *et al.*, 1993). The basis of this polyanion-dependent activation is presumably a change in charge at calcium-sensitive regulatory sites of the RyR. Such alterations caused by polyanions would lead to an increase in the local negative charge of the receptor complex and thus attract more calcium ions activating the receptor at its calcium-regulatory sites (Bezprozvanny *et al.*, 1993). The importance of the local charge around the pore of the RyR has been demonstrated by Tu *et al.* (1994). In that study, the importance of a negative surface charge for the potentiation of conduction and selectivity of the channel was shown. The effects described in the present paper also argue for changes in receptor charge as a possible mechanism of action. The RyR, which becomes activated by localized increases in calcium concentration near the channel (Smith *et al.*, 1986; Bezprozvanny *et al.*, 1991), would carry a higher positive charge caused by the binding of protamine. The effect of protamine on the calcium-activated channel RyR is independent of the calcium concentration on the cytosolic side of the RyR (Figure 3). The steep protamine concentration dependence of RyR activity (Figure 2D) indicates that many molecules of protamine bind to the RyR. These results imply that once a critical amount of protamine has bound to the RyR, as indicated by the sharply defined protamine concen-

Figure 4 (cont). the fluorescence changes measured (34 protamine-injected and 29 control-injected cells were analyzed). The application of the caffeine stimulus is indicated by arrows in D and E. Fluorescence intensity was measured as the ratio of fluorescence intensity during drug application (F) and the measured average baseline fluorescence intensity (F_0). (D) A control cell responded to the addition of calcium with a typical calcium transient (1 of 29). (E) Caffeine-induced increase in intracellular calcium concentration was not observed in cells that were injected with protamine (1 of 34). In independent experiments, protamine-injected cells were tested for intact intracellular calcium stores by stimulating the release of calcium from intracellular stores with thapsigargin. Thapsigargin-induced intracellular calcium release did not differ between control-injected (n = 32), untreated (n = 54), and protamine-injected cells (n = 45). A typical thapsigargin (5 μM) response of a protamine-injected cell is shown in F. Note that the time scales in D, E, and F are different.

tration that is needed to completely block the RyR, access of calcium ions to regulatory sites is prevented (Figure 2). The introduction of positive charges to the RyR complex could be interpreted as an indirect block of the channel by introducing cation-repelling positive charges close to the pore or to the cytosolic site that activates the RyR by binding calcium. The size of protamine makes a direct interaction with the channel pore unlikely, but allosteric effects on the receptor conformation cannot be excluded given the present data. This possible mechanism would contribute to the current view of RyR function. Direct interaction of the channel with the divalent cation calcium is sufficient to explain many basic regulatory aspects of the RyR. Taking into account the size of calcium ions, an alternative mechanism involving steric hindrance of calcium ions binding to the RyR by protamine is unlikely. However, reversible conformational changes of the RyR or of accessory proteins (Valdivia, 1998; MacKrill, 1999) induced by protamine cannot be excluded. Such changes could alter calcium regulatory sites. Further experiments to determine the effects of protamine binding on the RyR protein structure are needed, once structural data are available for the calcium-binding sites of the RyR (Chen *et al.*, 1992, 1993; Chen and MacLennan, 1994).

Blocking effects of highly positively charged molecules on the release of calcium from microsomes loaded with calcium have been reported (Palade, 1987). The present study confirms the results of this release study at the single-channel and cell level. The K_i values determined previously (Palade, 1987) for protamine activity in the presence of 10 mM caffeine to induce calcium release (9.8 ng/ml) are lower than the values obtained from the present single-channel studies (half-maximal inhibition at 37 ± 1 μg/ml). Such quantitative differences are possibly the result of differences in protamine and microsome preparations. The present half-maximal inhibition values observed for single-channel activity are in the same concentration range as those reported for a number of basic proteins inhibiting thymol-stimulated calcium release (Palade, 1987) and for heparin activating RyR channels (Bezprozvanny *et al.*, 1993).

The effect of protamine on the other main class of intracellular calcium channels, IP_3R, remains to be determined. Under our experimental conditions, the IP_3R was inactive because of a lack of its ligand in both the bilayer experiments and the caffeine-induced calcium release experiments in intact cells. Studies of the antagonistic effects of heparin on IP_3Rs and RyRs (Supattapone *et al.*, 1988; Bezprozvanny *et al.*, 1993) and calcium release studies (Palade, 1987, Palade *et al.*, 1989), together with the present data, should stimulate future studies on the effects of protamine on IP_3Rs.

Protamine is a widely used heparin antidote in surgery and has been associated with hypotension in patients (Ordonez Fernandez *et al.*, 1998). Protamine was found to have potentially direct and indirect effects by influencing intercellular and intracellular signaling pathways (Akata *et al.*, 1991; Ordonez Fernandez *et al.*, 1998). The present study provides evidence that once protamine is allowed to enter cells and to have access to RyRs, the effect on RyR-mediated calcium release is significant. Additional experiments are required to determine if an uptake of protamine into muscle and endothelial cells is possible and if pathological conditions could be involved in such a process.

The present study shows that the described inactivation of RyRs by protamine at the single-channel level can be used in living cells. This is important for the evaluation of single-channel data in that the receptor also shows the described modulation in its native environment. Physiological assays involving the function of the RyR could make use of the described channel inhibition. The block of RyR-mediated intracellular calcium release by protamine can be used in a variety of cells to determine the contribution of RyRs to cellular processes (Ehrlich and Bezprozvanny, 1994). RyR-mediated calcium signaling plays an important role in the development of cells and organs (Ferrari and Spitzer, 1999). Microinjection of protamine, as shown in the present study, could be used to selectively but also reversibly block RyRs and their action in developing cells. With this new tool, it will be possible to determine the exact time courses of RyR function during development.

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