The effect of the PKC inhibitor calphostin C and the PKC agonist phorbol 12-myristate 13-acetate on regulation of cytosolic Ca\(^{2+}\) in mammalian skeletal muscle cells

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Received 16 June 2005; revised 29 July 2005; accepted 31 July 2005
Available online 8 September 2006

Abstract

Protein kinase C (PKC) has been shown to exert broad actions in modulating Ca\(^{2+}\) in cardiac myocytes, however, the effect of PKC in skeletal muscle cells is largely unknown. In this study, we examined the effect of the PKC inhibitor calphostin C (CC) and the PKC agonist phorbol 12-myristate 13-acetate (PMA) on intracellular Ca\(^{2+}\) handling in C2C12 skeletal myotubes and skinned skeletal muscle fibers of the rat. CC (250 nM) significantly prolonged (P = 0.01, n = 6), and the PKC agonist PMA (500 nM; P = 0.03, n = 6) significantly shortened the decay phase of electrically induced Ca\(^{2+}\) transients in C2C12 myotubes without affecting the amplitude or the time to peak of the transients. Skinned fiber studies showed that CC significantly inhibits SR Ca\(^{2+}\) uptake in skeletal muscle cells. PMA had no effect. CC also increased the peak of ATP-induced Ca\(^{2+}\) transients release by 94.2% (P < 0.0001) in the presence of extracellular Ca\(^{2+}\) and 54.5% (P = 0.04) without external Ca\(^{2+}\) via IP\(_3\)-Ca\(^{2+}\) release pathway in C2C12 myotubes, while PMA had no effect, suggesting that CC may modulate IP\(_3\)-induced Ca\(^{2+}\) release via a PKC-independent mechanism. CC at a concentration of 1 \(\mu\)M was able to induce a large sustained elevation in basal [Ca\(^{2+}\)]i that was blocked by Ca\(^{2+}\) store depletion and the IP\(_3\) receptor blocker 2-APB. These results indicate that PKC plays a role in modulation of SR function in skeletal muscle cells, and the PKC inhibitor CC may alter Ca\(^{2+}\) handling via both PKC-dependent and PKC-independent pathways.

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Keywords: Calphostin C; Ca\(^{2+}\); PKC-independent; Sarcoplasmic reticulum; Skeletal muscle

Introduction

Protein kinase C (PKC) is a serine/threonine kinase that was first characterized based on its activation in vitro by Ca\(^{2+}\) and phospholipid and diacylglycerol (DAG) (Nishizuka, 1992; Asaoka et al., 1992). PKC is translocated to the membranes upon activation, and thus the membrane-associated kinase is considered to be the active form. PKC activation may play an important role in skeletal muscle function as both Ca\(^{2+}\)-dependent (PKC-\(\alpha\)) and Ca\(^{2+}\)-independent (PKC-\(\theta\)) PKC isoforms have been shown to be enriched in skeletal muscle (Osada et al., 1992), and total PKC translocation is markedly enhanced by electrically activated contractile activity in skeletal muscle (Richter et al., 1987).

The involvement of PKC in intracellular signaling process is usually undertaken using phorbol esters, which mimic DAG and activate PKC (Blumberg, 1998), or PKC inhibitors such as calphostin C (CC). CC is a substance originally isolated from the fungus Cladosporium cladosporioides. CC is reported to be a selective and potent inhibitor of PKC (IC\(_{50}\) = 50 nM) (Kobayashi et al., 1989) and is unusual in that it is activated by light, with strong absorbance in the visible and ultraviolet ranges (Bruns et al., 1991). CC is thought to competitively bind PKC by covalent attachment to the DAG binding site (Tamaoki and Nakano, 1990) and then damage the enzyme via light-dependent free radical production (Kobayashi et al., 1989).
Little is known about the action of PKC in the modulation of SR function and free cytosolic Ca\(^{2+}\) in skeletal muscle cells. However, in cardiac myocytes, CC is reported to increase the peak and slow the decay of the [Ca\(^{2+}\)]\(_i\) transients and elevate the basal cytosolic [Ca\(^{2+}\)] which may arise from the ability of CC to slow SR Ca\(^{2+}\) uptake in these cells through PKC inhibition (Nicolás et al., 1998).

Strict control of cytosolic Ca\(^{2+}\) is critical in skeletal muscle cells as Ca\(^{2+}\) plays a pivotal role in excitation–contraction coupling (ECC). ECC is the complex series of events that link depolarization of sarclemma to activation of contraction. Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) though ryanodine receptors (RyR) is primarily responsible for this process in skeletal muscle, and the resulting rapid increase in cytosolic Ca\(^{2+}\) promotes Ca\(^{2+}\)-mediated activation of tropomysin C which removes the inhibitory factors and promotes cross-bridge cycling (Lamb, 2000). However, in skeletal muscle, increases in cytosolic Ca\(^{2+}\) can also be brought about by IP\(_3\)-induced Ca\(^{2+}\) release from the SR through activation of IP\(_3\) receptors (Volpe et al., 1986) and activation of sarcolemmal Ca\(^{2+}\) channels by electrical excitation, ligand binding or depletion of the intracellular Ca\(^{2+}\) store (store-operated Ca\(^{2+}\) entry) (Gailly, 2002). Elevated intracellular Ca\(^{2+}\) is lowered in skeletal muscle by powerful Ca\(^{2+}\) pumps located in the SR membrane and Ca\(^{2+}\) pumps and exchangers located in the sarclemma.

Abnormal Ca\(^{2+}\) regulation involving any of these sites in skeletal muscle cells may result in defective muscle contraction and/or relaxation, as well as muscle degeneration (Johnson and Bhattacharya, 1993). Altered Ca\(^{2+}\) signaling has been implicated in many muscular disorders such as malignant hyperthermia (Michelson and Louis, 1996), central core disease (Froemming and Ohlendieck, 2001) and Duchenne muscular dystrophy (Bodensteiner and Engel, 1978).

The aim of the present study was to investigate the role of PKC on different aspects of Ca\(^{2+}\) handling and SR function in isolated adult and cultured skeletal muscle cells using the PKC inhibitor CC and the PKC agonist phorbol 12-myristate 13-acetate (PMA).

**Methods**

All the procedures used in this study were in accordance with the guidelines and an approved protocol of the University of Western Australia Animal Ethics Committee.

**C2C12 cultures and myotube differentiation.** The C2C12 myoblasts were grown in culture flasks containing Dulbecco’s modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO), 2.0 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% fungizone in a humidified atmosphere of 5% CO\(_2\) and 95% O\(_2\) at 37 °C. To produce myotubes, trypsinized myoblasts were transferred to collagen-coated coverslips placed in Petri dishes and grown in the same culture medium and conditions as described above. When they were 60–70% confluent, the 10% fetal bovine serum was replaced by 2% horse serum (GIBCO) to encourage fusion of the myoblasts into myotubes. Spindle-shaped cells in the first stages of differentiation to myotubes were observed after 24 h of incubation. Within 3 to 6 days, the cultured cells grew into contractile myotubes, which were used in this study.

**Measurement of [Ca\(^{2+}\)]\(_i\).** The intracellular Ca\(^{2+}\) concentration was monitored in C2C12 myotubes using Fura-2 and dual excitation wavelength fluorescence microscopy. Cells were loaded with Fura-2 by incubation for 45 min (room temperature) in physiological rodent saline solution (PRS; composition in mM: 138 NaCl, 2.7 KCl, 1.8 CaCl\(_2\), 1.06 MgCl\(_2\), 12.4 HEPES and 5.6 glucose, pH 7.3) containing 3 µM Fura-2/AM and 0.0125% (w/v) Pluronic F-127 (total DMSO: ~0.6% v/v) (Molecular Probes, Eugene, OR). The Fura-2-loaded myotubes were washed three times with fresh PRS and were placed in a chamber (total volume ~2 ml) mounted onto the stage of a Nikon TE2000 inverted microscope. With light provided by a Cairn dual monochromator system (Cairn, UK), the cells were illuminated alternatively at excitation wavelengths of 340 and 380 nm (bandwidth = 10 nm), and the Fura-2 fluorescence emission at 510 nm (20 nm bandpass) was acquired. To limit the detection of emitted fluorescence to that emitted from a single cell, an adjustable optical sampling window was positioned within the light path upstream from the photomultiplier tube. Data were analyzed using the Cairn software package (Cairn, UK). Electrically induced Ca\(^{2+}\) transients were elicited using electrical field stimulation (EFS) (supramaximal, 0.5-ms rectangular pulse) and recorded at the maximal time resolution for the Cairn spectrophotometer system (100 Hz). Slower changes in basal Ca\(^{2+}\) were monitored at a sampling rate of 5 Hz. The fluorescence ratio at 340- and 380-nm excitation (F\(_{340}\)/F\(_{380}\)) was used as an indicator of changes in cytosolic Ca\(^{2+}\) concentration. The maximal fluorescence ratio possible under saturating levels of Ca\(^{2+}\) was determined by exposing β-escin-permeabilized C2C12 cells to 2 mM external Ca\(^{2+}\) and was found to be 2.75 ± 0.20 (n = 5). The experiments were performed in the presence of the myosin inhibitor N-Benzyl-P-toluene sulfonamide (50 M) (Sigma), to prevent activation of the contractile filaments by the elevated Ca\(^{2+}\). This drug does not affect Ca\(^{2+}\) determination with fluorescent indicators (Cheung et al., 2002).

**Examining the effect of CC on the SR function.** The effect of CC on the SR function was specifically investigated using the skinned fiber technique. Skeletal muscle fibers were isolated from the extensor digitorum longus (EDL) muscles of Wistar rats (~400 g), killed by exposure to a gas mixture of 80% CO\(_2\) and 20% O\(_2\). The single muscle fibers were dissected and mechanically skinned in paraffin oil. To monitor isometric force, the fibers were mounted between a fixed pair of forceps and a force transducer and
placed in a Perspex bath containing 2 ml of a K+–HDTA solution (mM): K+; 125; Na+, 36; HDTA2−, 50; ATP (total), 8; Mg2+ (total), 8.6; creatine phosphate, 10; EGTA (total), 0.03; HEPES, 90; NaN3, 1 at pH 7.10 ± 0.01 (Lamb and Stephenson, 1990). The free Mg2+ concentration was 1 mM. NaN3 was added to inhibit mitochondrial Ca2+ fluxes. Data were acquired using a PowerLab data acquisition system (AD Instruments) attached to a PC. To maximize the force production, the fibers were stretched from slack length by 20% to bring the sarcomere length to approximately 2.8–3.0 μm (Lamb and Stephenson, 1990).

The experiments to investigate the effect of CC on SR Ca2+ release and SR Ca2+ loading were similar as described previously (Han and Bakker, 2003; Han et al., 2003). Briefly, the fibers were firstly depleted of Ca2+ by exposure for 2 min to a maximal Ca2+ release solution (K+–HDTA solution with 30 mM caffeine and 0.25 mM EGTA). The fiber was then reloaded with Ca2+ for 10 s by exposure to a highly [Ca2+] buffered solution (pCa 6.55). Following this, the fiber was washed in the K+–HDTA solution to remove excess EGTA and then exposed to a submaximal Ca2+ release solution (K+–HDTA with 20 mM caffeine) with or without CC (250 nM) or PMA (500 nM) and the force response measured and compared to control measurements made before and after drug exposure.

The experiments to determine the effect of CC and PMA on SR Ca2+ loading were similar to the SR Ca2+ release experiments as described above with the exception that the drug (CC or PMA) was present in the load solution but not in the release solution. After loading, the fibers were exposed to the depletion solution again to release all loaded Ca2+, and the integral of the force response was used as an indicator of the amount of Ca2+ loaded during the loading period (Han and Bakker, 2003; Han et al., 2003).

Statistical analysis. Data are presented as means ± SEM. The “n” number is the number of cells examined. Where appropriate, the significance of differences between experimental groups was assessed using Student’s t test (paired or unpaired, or one sample test). P < 0.05 was regarded as significant. The traces shown in the figures are representative single experiments.

Results

The effect of CC on Ca2+ release and uptake

To study the effect of CC on Ca2+ release and uptake during ECC, EFS-induced Ca2+ transients were elicited in mature myotubes, before and after exposure to CC. Under control conditions, the rise time of the Ca2+ transients was 25.4 ± 4.5 ms, and the half relaxation time was 340 ± 27 ms (n = 12). CC (250 nM) significantly increased the half relaxation time of the transients to 405 ± 47 ms (P = 0.01) but did not have any significant effect on the peak (P = 0.83) or the time to peak (P = 0.64) of the Ca2+ transients after 5-min pre-incubation in CC (n = 6) (Fig. 1a). On the other hand, the PKC agonist PMA (500 nM) significantly decreased the half relaxation time of the Ca2+ transients to 316 ± 42 ms (P = 0.03) after 5-min pre-incubation but also had no significant effect on the peak (P = 0.15) or the time to peak (P = 0.36) of the Ca2+ transients (n = 6) (Fig. 1b).

It is possible that the maximal sampling rate of the spectrophotometer system (100 Hz) might be too slow to accurately track the rising and peak phases of the Ca2+ transients. Therefore, in order to provide further information about the effects of CC on SR Ca2+ release, the effect of CC on caffeine-elicited (20 mM) Ca2+ release was examined in skinned adult skeletal muscle fibers of the rat.

To examine the effect of CC on SR Ca2+ release, skinned fibers were initially treated with a maximum caffeine release solution (with EGTA) to deplete all releasable Ca2+ from the SR. The fibers were then loaded with Ca2+ under control conditions, and the effect of CC on SR Ca2+ release was examined. CC (250 nM) had no significant effect on the peak of the caffeine-induced force responses compared to the mean control responses made both before and after SR Ca2+ release responses in the presence of CC (CC: 98.6 ± 2.4% of the control responses; P = 0.58, one-sample Student’s t test, n = 6) (Fig. 2a). This finding is consistent with the lack of effect of CC on the Ca2+ transients in the myotubes, and, taken together, these data suggest that CC has no significant effect on ryanodine receptors in skinned adult skeletal muscle fibers.

The effects of CC on Ca2+ transient decay suggest a putative inhibitory role of CC on SR Ca2+ pump activity.
In order to verify this finding, the effect of CC on SR Ca\textsuperscript{2+} loading was directly investigated in adult skeletal muscle fibers using the skinned fiber technique (Bakker and Berg, 2002; Han and Bakker, 2003; Han et al., 2003). CC was found to significantly decrease SR Ca\textsuperscript{2+} loading to 93.3 ± 1.6% (P = 0.01, n = 5) of control levels (mean of control measurements made before and after exposure to CC) in skinned EDL fibers (Fig. 2b), indicating that CC can inhibit the SR Ca\textsuperscript{2+} uptake in skeletal muscle fibers.

In order to provide more information about whether the effect of CC on SR function is via inhibition of PKC or a PKC-independent effect, the effect of PMA on SR function was also studied in skinned fibers. PMA (500 nM) had no significant effect on either the peak of the caffeine-induced force responses (97.2 ± 3.5% of the control responses; P = 0.79, n = 8) or SR Ca\textsuperscript{2+} loading (98.7 ± 1.9 of control levels %, P = 0.82, n = 7) in skinned EDL fibers (data not shown).

The effect of CC on ATP-induced Ca\textsuperscript{2+} transients in C2C12 myotubes

In order to gain insight into the possible modulation of IP\textsubscript{3} receptors-mediated Ca\textsuperscript{2+} release from the SR by CC, as opposed to RyR-mediated SR Ca\textsuperscript{2+} release, the effect of CC on ATP-induced Ca\textsuperscript{2+} transients in C2C12 myotubes was also examined.

Extracellular application of ATP (300 μM) in differentiated C2C12 myotubes elicited a marked elevation in intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}])\textsubscript{i} that rose within seconds and was followed by a slower decay phase (Fig. 3). The elevation of intracellular Ca\textsuperscript{2+} induced by ATP in C2C12 myotubes is reported to be mediated by both IP\textsubscript{3}-induced Ca\textsuperscript{2+} release via activation of phospholipase-C-coupled P\textsubscript{2Y\textsubscript{2}}-purinoceptors (or recently termed P\textsubscript{2Y\textsubscript{2}} receptors) (Henning et al., 1993). The maximal amplitude of the ATP-induced Ca\textsuperscript{2+} transients was decreased in nominal Ca\textsuperscript{2+}-free solution from 0.428 ± 0.025 (n = 13) to 0.287 ± 0.033 (n = 9) (P = 0.003, unpaired Student’s t test) (Fig. 3). These observations are consistent with previous studies (Henning et al., 1993, 1996) and indicate that ATP induces both SR Ca\textsuperscript{2+} release and extracellular Ca\textsuperscript{2+} influx. In order to examine the effect of CC on ATP-induced Ca\textsuperscript{2+} release alone, the effect 250 nM CC on ATP-induced Ca\textsuperscript{2+} transients was undertaken, while the cells were bathed in nominally Ca\textsuperscript{2+}-free saline. Under these conditions CC markedly increased the peak of the ATP-induced Ca\textsuperscript{2+} transients from a mean fluorescence ratio of 0.287 ± 0.033 (n = 9) to 0.415 ± 0.045 (n = 9), an increase of 54.5% (unpaired Student’s t test, P = 0.04) (Figs. 3a, b). In the presence of external Ca\textsuperscript{2+}, CC also markedly increased the amplitude of the ATP-induced Ca\textsuperscript{2+} transients from a mean ratio of 0.428 ± 0.025 (n = 13) to 0.822 ± 0.051 (n = 6), an increase of 94.2%, (unpaired Student’s t test, P < 0.0001) The difference in the half-decay time (the time from the peak to the half peak during decay phase, τ) of the ATP-induced Ca\textsuperscript{2+} transients before and after CC pretreatment was not statistically significant (P = 0.79 and P = 0.17 under Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+} present conditions respectively), and it was extremely variable from cell to cell under all conditions (range: 30 s to 355 s).

To examine the possible involvement of PKC in the effect of CC, the effect of PMA on ATP-induced Ca\textsuperscript{2+} responses was also investigated. Pretreatment with 500 nM PMA for 10 min had no significant effect on the peak of the ATP-induced Ca\textsuperscript{2+} responses in the presence of normal (peak increase: 0.407 ± 0.106, n = 5, P = 0.80, unpaired Student’s t test) or low external Ca\textsuperscript{2+} (peak increase: 0.365 ± 0.092, n = 4, P = 0.33, unpaired Student’s t test) (Figs. 3c, d). Nor did pretreatment with PMA significantly affect the half-decay time of the ATP-induced Ca\textsuperscript{2+} transients (P = 0.12 and 0.07 in the presence of normal and low extracellular Ca\textsuperscript{2+} respectively). These results suggest that the effect of CC on ATP-induced intracellular Ca\textsuperscript{2+} release may not be related to inhibition of PKC.

The effect of CC on resting [Ca\textsuperscript{2+}]\textsubscript{i} in C2C12 myotubes

The effect of CC on the basal cytosolic Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]) in mature C2C12 myotubes (3–6 days old in differentiation media) was also examined. PKC inhibitory concentrations of CC (≤ 250 nM) had no significant effect on the basal (unstimulated) [Ca\textsuperscript{2+}]\textsubscript{i} in the myotubes after 15 min exposure (n = 10). However, when CC was applied at 1 μM, the resting fluorescence ratio (R\textsubscript{340/380}) significantly increased by 56.3 ± 4.5% within 10 min compared to the...
control ratio before CC was applied (control, 0.601 ± 0.037; 10 min after CC, 0.939 ± 0.054; \( P < 0.0001, n = 11 \)) (Fig. 4a).

The resting ratio did not return to the baseline level after CC application. Exposure of the myotubes to a similar amount of the vehicle alone (0.1% DMSO, v/v) had a very small but significant elevation (3.8% increase of the ratio after 10 min) on \([\text{Ca}^{2+}]_i\) (\( P = 0.03, n = 6 \)) (Fig. 4b). Correcting for the small effect of DMSO, the increase in fluorescence ratio produced by CC (1 \(\mu\)M) was approximately 52.5%.

Microscopic visualization of the cells after prolonged exposure to CC (1 \(\mu\)M) for 10–15 min revealed that the normally elongated myotubes had become roughly spherical, indicating that a marked cell shortening had occurred. This observation is consistent with the CC-induced elevation in intracellular \([\text{Ca}^{2+}]_i\) causing activation of the contractile apparatus and a large contraction. This is likely to have ruptured myotubes cell adhesion/anchorage points on the substrate leading to cell shortening and a large change on morphology.

The PKC inhibitor staurosporine had no observable effect on \([\text{Ca}^{2+}]_i\) during 15 min measured at a concentration that should fully inhibit PKC (20 nM). A very high concentration of staurosporine (1 \(\mu\)M, \( n = 3 \); 5 \(\mu\)M, \( n = 6 \)) did appear to increase the basal \([\text{Ca}^{2+}]_i\) in the myotubes. However, at these concentrations, staurosporine altered the fluorescence properties of Fura-2 in the myotubes, increasing the fluorescence intensities at both excitation wavelengths and making the extent of the \([\text{Ca}^{2+}]_i\) increase induced by this drug unreliable (data not shown). Another PKC inhibitor chelerythrine was also tested but was again unusable due to strong autofluorescence.

PMA (1 \(\mu\)M, with 0.1% (v/v) DMSO) did not significantly affect the basal \([\text{Ca}^{2+}]_i\) (Fig. 4c). The resting ratio was 0.608 ± 0.043 after 15 min of incubation with 1 \(\mu\)M PMA.
compared with the pre-control ratio of 0.594 ± 0.039 (P = 0.31, n = 3). However, the effect of PMA on [Ca^{2+}]_i might have been underestimated because of the 3.8% increase on [Ca^{2+}]_i by the vehicle DMSO.

In order to explore the source of Ca^{2+} for the CC-induced Ca^{2+} elevation, the effect of CC on [Ca^{2+}]_i was conducted both under the conditions of low extracellular Ca^{2+} ([Ca^{2+}]_o) and in the presence of the Ca^{2+} channel blockers, La^{3+} or Ni^{2+}. The basal intracellular Ca^{2+} level was slightly decreased after exposure to a nominal Ca^{2+}-free rodent saline (Ca^{2+} was replaced with 3.6 mM Mg^{2+} to minimize charge screening effects of Ca^{2+} removal, and 1 mM EGTA was added to chelate Ca^{2+}) for 10 min (control conditions, 0.604 ± 0.021, n = 31; Ca^{2+}-free conditions, 0.572 ± 0.023, n = 14; P = 0.04, unpaired Student’s t test), indicating that there was a basal Ca^{2+} influx in these cells. Under Ca^{2+}-free conditions, 1 μM CC still increased [Ca^{2+}]_i by 37.2 ± 0.3% (paired Student’s t test, P < 0.0001, n = 3) after 10 min (Fig. 5). Furthermore, pretreatment of the cells with 0.5 mM La^{3+} or 2 mM Ni^{2+} for 3 min did not block the elevation of [Ca^{2+}]_i induced by CC (Data not shown). These results indicate that CC-induced Ca^{2+} elevation is predominantly due to the release of Ca^{2+} from an intracellular site.

In order to determine whether the intracellular site of Ca^{2+} release elicited by CC was the SR, we used the SR Ca^{2+} pump inhibitor 2,5-di-(tert-butyl)-1,4-dihydroquinone (TBQ) (30 μM) (Bakker et al., 1996) to deplete the SR of Ca^{2+} before application of CC. Application of TBQ to the myotubes produced a large increase in cytosolic Ca^{2+} followed by a slow Ca^{2+} decline until the [Ca^{2+}]_i eventually returned to the resting level after around 10–15 min. This effect is typical of Ca^{2+} store pump inhibitors (e.g. thapsigargin) and is thought to be due to the net leak of Ca^{2+} from the Ca^{2+} store in the absence of store Ca^{2+} uptake. Ca^{2+} declines due to the action of sarcolemmal Ca^{2+} extrusion mechanisms (Thomas and Hanley, 1994) (Fig. 6). At approximately 15–20 min after TBQ administration, 1 μM CC was added in the bath solution. Administration of CC for 10–15 min did not increase the [Ca^{2+}]_i in TBQ-pretreated cells in the presence of physiological or low extracellular concentrations of Ca^{2+} in the bath solution (P = 0.32, n = 5) (Fig. 6). This result indicates that Ca^{2+} release from the SR is responsible for the effect of CC on the basal Ca^{2+} in the C2C12 myotubes.

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**Role of ryanodine receptors and IP3 receptors on the CC-induced Ca^{2+} response**

Both ryanodine-sensitive and IP3-sensitive SR Ca^{2+} release mechanisms have been shown to be present in adult skeletal muscle (Talon et al., 1999, 2002) and cultured primary and C2C12 myotubes (Jaimovich et al., 2000; Powell et al., 2001). An examination into the pathway responsible for the CC-induced SR Ca^{2+} release was undertaken using the ryanodine receptor inhibitor dantrolene (Krause et al., 2004; Wright et al., 2005) and the IP3 receptor inhibitor 2-aminoethoxydiphenyl borate (2-APB) (Ma et al., 2000; Maruyama et al., 1997).

As shown in Fig. 7a, in the presence of dantrolene (20 μM), addition of 1 μM CC still increased the basal cytosolic Ca^{2+} level by 135.8 ± 8.6% (n = 9), which was not significantly different to the Ca^{2+} increase produced by CC alone (134.3 ± 5.1%, n = 5, P = 0.90). Exposure to dantrolene alone for a similar time period did not alter the basal Ca^{2+} levels in the myotubes (n = 3, data not shown). This result indicates that CC is not increasing basal cytosolic Ca^{2+} in the myotubes by inducing Ca^{2+} releases from the intracellular Ca^{2+} store via activation of ryanodine receptors.

In this study, application of the IP3 receptor blocker 2-APB (50 μM) itself elicited an increase in [Ca^{2+}]_i, in the myotubes, and this [Ca^{2+}]_i elevation returned to near baseline condition within ~10 min (Fig. 7b). In 3 cells which had been exposed to 50 μM 2-APB and Ca^{2+} had returned to baseline, 1 μM CC produced no increase in [Ca^{2+}]_i after 15 min (Fig. 7b), suggesting inhibition of the CC-mediated effect. However, as 2-APB itself caused a small increase in basal Ca^{2+} (Fig. 7b), the possibility arises that the absence of an effect of CC on basal Ca^{2+} may have been due to 2-APB-mediated depletion of the SR of Ca^{2+}, and not blockage of the IP3 receptors. To investigate this possibility, we exposed myotubes to a Ca^{2+} release solution after prolonged incubation in 2-APB. The Ca^{2+} release solution contained caffeine (5 mM), which releases Ca^{2+} from the SR via the ryanodine receptors, and TBQ (30 μM), which blocks Ca^{2+} reuptake via the SR Ca^{2+} pumps (Bakker et al., 1996). From Fig. 7c, it can be seen that exposure of the myotubes to the Ca^{2+} release solution after 2-APB
Discussion

This study shows that CC has marked effects on Ca^{2+} handling in skeletal muscle cells. One of the most important results of this study is that CC was able to inhibit SR Ca^{2+} uptake in skeletal muscle fibers, which explains the slower decline of the EFS-induced Ca^{2+} transients measured in the myotubes in the presence of CC. The observation that the PKC agonist PMA had the opposite effect to CC and increased the decay rate of the EFS-elicited Ca^{2+} transients in C2C12 myotubes indicates that the effect of CC on SR Ca^{2+} uptake is PKC-mediated. These results indicate that PKC is involved in regulation of SR Ca^{2+} uptake in skeletal muscle cells, as it is in cardiac muscle (Nicolas et al., 1998).

Drugs that specifically inhibit SR Ca^{2+} uptake such as TBQ have been shown to increase SR Ca^{2+} release, as under normal circumstances, the ongoing SR Ca^{2+} uptake dampens SR Ca^{2+} release to some extent (Bakker et al., 1996). However, in this study, CC had no effect on the peak of caffeine-induced force responses in the skinned fibers nor did CC increase the peak of the EFS-elicited Ca^{2+} transients in the myotubes. Taken together, these results suggest that the ryanodine receptors are unaffected by calphostin C and that the moderate inhibition of SR Ca^{2+} loading produced by 250 nM CC (93% of control uptake in the skinned fibers) may be insufficient to substantially alter the amount of Ca^{2+} released from the SR during activation of these cells.

In contrast to the effect of PMA on the Ca^{2+} transients elicited in the myotubes, PMA did not increase SR loading in the skinned fibers. However, the lack of effect of PMA in the skinned fibers may be due to the nature of its action. PMA mimics diacylglycerol, the native activator of PKC, which works by initiating translocation of PKC to the membrane, thereby activating the enzyme (Slater et al., 2002). In the skinned fibers, much of the freely diffusible PKC may be washed away and not present for translocation to the membrane, and hence PKC activity is not substantially enhanced.

This study also investigated the effects of CC on ATP-induced Ca^{2+} transients in C2C12 myotubes. CC was found to markedly increase the peak of ATP-induced Ca^{2+} responses in C2C12 myotubes in the absence of external Ca^{2+}, where the Ca^{2+} signal should be almost entirely due to IP_{3}-mediated Ca^{2+} release from the SR (Henning et al., 1993). PMA, however, had no significant effect on the peak of the Ca^{2+} response to ATP, suggesting that CC may increase the activity of the IP_{3} receptors in the C2C12 myotubes via a PKC-independent mechanism. Alternatively, it is possible that the PKC is involved and PMA has little effect because PKC activation is already maximal in these cells.

In this study, continuous exposure to a higher CC concentration (1 mM) was found to lead to a slow, large and sustained increase in [Ca^{2+}], in the C2C12 myotubes. These Ca^{2+} responses were as large in magnitude as those induced by ATP. Unlike the ATP-mediated Ca^{2+} signals, the 1 mM CC-induced Ca^{2+} elevation did not decline, and, after 15 min, the myotubes underwent marked changes in morphology consistent with hyper-contracture and, possibly, cell death.

The mechanism responsible for this effect was explored in this study and was shown to be due to the release of Ca^{2+} from an intracellular source as removal of extracellular Ca^{2+} did not prevent the [Ca^{2+}], increase induced by CC. The responses were abolished by pretreatment with the SR Ca^{2+} pump inhibitor TBQ, which is known to specifically deplete the Ca^{2+} store of Ca^{2+}, indicating that the SR is the source of released Ca^{2+} induced by CC application. A number of pharmacological approaches were employed to identify the molecular mechanisms involved in the response of the cells to CC. Application of the ryanodine receptor inhibitor dantro-
lune (20 μM) failed to prevent CC-induced Ca\(^{2+}\) mobilization, suggesting that CC did not promote Ca\(^{2+}\) release via ryanodine receptors. This result is also consistent with the negligible effects of CC on EFS-induced Ca\(^{2+}\) transients in the myotubes and the caffeine-induced responses elicited in the skinned fibers, both of which due to Ca\(^{2+}\) release via the ryanodine receptors. However, the CC-induced elevation of basal Ca\(^{2+}\) was completely blocked by the membrane-permeable IP\(_3\) receptor blocker 2-APB (Ma et al., 2000; Maruyama et al., 1997). While 2-APB (50 μM) itself induced a Ca\(^{2+}\) elevation, a caffeine/TBQ-based Ca\(^{2+}\) release solution was still able to induce a significant Ca\(^{2+}\) response after pretreatment with 2-APB, indicating that 2-APB (50 μM) did not deplete the SR Ca\(^{2+}\) store in our study. Therefore, the absence of an effect of CC after 2-APB treatment cannot be simply attributed to SR Ca\(^{2+}\) depletion by 2-APB.

These results suggest that the sustained elevation in Ca\(^{2+}\) elicited by 1 μM CC in the C2C12 myotubes is due to CC-mediated activation of the IP\(_3\)-mediated Ca\(^{2+}\) release pathway. The fact that neither a lower concentration of CC (250 nM) nor the PKC inhibitor staurosporine (20 nM) induced a [Ca\(^{2+}\)]\_i elevation within a similar time frame, even though they should both substantially inhibit PKC at these concentrations, and that PMA was without effect suggests that the CC (1 μM)-induced Ca\(^{2+}\) response is likely to be PKC-independent. However, the PKC-dependent slowing of SR Ca\(^{2+}\) uptake shown in this study (in the EFS-induced Ca\(^{2+}\) transients and skinned fiber measurements) would presumably enhance the sustained Ca\(^{2+}\) release through the IP\(_3\) receptors elicited by 1 μM CC and may be partly responsible for the absence of any noticeable decline in the Ca\(^{2+}\) signal after exposure to 1 μM CC. C2C12 myotubes mainly express the type 3 IP\(_3\) receptor isoform (Powell et al., 2001). IP\(_3\) receptors are stimulated by Ca\(^{2+}\), and hence CC-induced IP\(_3\) receptor-mediated Ca\(^{2+}\) release should be further enhanced as Ca\(^{2+}\) release ensues. However, the type 3 IP\(_3\) receptors, unlike the type 1 IP\(_3\) receptors, are not reported to be inhibited by μM Ca\(^{2+}\) levels (Thrower et al., 2001), which would further help to explain the lack of decline in the Ca\(^{2+}\) signal after exposure to 1 μM CC.

PKC-independent effects of CC have been described in previous studies. CC is reported to cause cell death by both apoptosis (Ikemoto et al., 1995; Jarvis et al., 1994) and necrosis (Larue et al., 2000), although the mechanism remains undefined. A recent study has shown that CC, but not other PKC inhibitors, induces rapid apoptosis in human acute lymphoblastic leukemia cells. CC also induced rapid calcium mobilization from intracellular Ca\(^{2+}\) stores in these cells, and the cytotoxic activity of CC was correlated with the magnitude of the CC-induced Ca\(^{2+}\) signal (Zhu et al., 1998). If similar pathways occur in myotubes and lymphoblastic leukemia cells, the combined effect of increased Ca\(^{2+}\) release via IP\(_3\) receptors and decreased Ca\(^{2+}\) uptake by the Ca\(^{2+}\) store could be responsible for promoting the cell death observed in these studies.

The results of this study indicate that PKC activation during skeletal muscle contraction may be an important modulator of SR function. This study also showed that the PKC inhibitor CC may have PKC-independent effects on intracellular Ca\(^{2+}\) signaling in skeletal muscle cells.

Acknowledgments

This work was supported by a grant from The Raine Medical Research Foundation. R. Han was a recipient of the International Postgraduate Research Scholarship and University Postgraduate Award from the University of Western Australia.

References


