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ORIGINAL ARTICLE

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Store operated Ca^{2+} influx by selective depletion of ryanodine sensitive Ca^{2+} pools in primary human skeletal muscle cells

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Abstract The contraction and relaxation of skeletal muscle is driven by release of Ca^{2+} from sarcoplasmic reticulum through the ryanodine receptor type 1 and extruding the ion from the cytosol by Ca^{2+}ATPases. Efficient refilling of the empty Ca^{2+} stores is essential for repetitive cycles of muscle contraction and relaxation, but not investigated in human skeletal muscle cells. Here we show that under conditions of selective depletion of the ryanodine-sensitive Ca^{2+} pool Ca^{2+} influx occurs in differentiated human skeletal muscle cells using the Ca^{2+} imaging technique. This Ca^{2+} influx is not due to permeation through the L-type Ca^{2+} channel and not observed under conditions of inhibited Ca^{2+} ATPase. The Ca^{2+} influx was visualised by quenching the intracellular fura2 signal with Mn^{2+} on single cell level and also using fluorescence photometry of cell suspensions. The Mn^{2+} influx was inhibited by the Ca^{2+} channel blockers La^{3+} and SKF96356. The delineation of the signalling cascade leading to Ca^{2+} influx evoked by selective depletion of ryanodine sensitive Ca^{2+} stores showed that phospholipase C or protein kinase C were not involved. Interestingly, a Mn^{2+} influx was triggered by the cell-permeant analogue of diacylglycerol and further augmented by the application of RHC80267, a diacylglycerol lipase inhibitor. This signalling pathway could be attributed to the participation of a protein kinase C activity. However, Mn^{2+} influx evoked by selective depletion of ryanodine sensitive Ca^{2+} stores was not altered by RHC80267 or protein kinase C inhibitors. Using RT-PCR, correctly spliced mRNA fragments were detected corresponding to human transient receptor potential (TRPC) Ca^{2+} channels type 1, 3, 4 and 6. These data show that in skeletal muscle at least two independent mechanisms of Ca^{2+} influx exist. For Ca^{2+} influx triggered by the selective depletion of ryanodine sensitive Ca^{2+} stores we propose a phospholipase C independent coupling of ryanodine receptors to voltage insensitive Ca^{2+} channels.

Keywords Skeletal muscle · Ryanodine receptor · Store-operated Ca^{2+} influx · Diacylglycerol · Protein kinase C · Sarcoplasmic reticulum

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Introduction

Ca^{2+} is a universal second messenger which triggers short term cellular responses (e.g. contraction in heart and skeletal muscle) as well as long term cellular adaptive responses (e.g. T-lymphocyte reactivity or long term potentiation in neuronal cells) (Berridge 1997; Berridge et al. 1998; Clapham 1995). The intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, is modulated by two sources, influx from the extracellular space and release from intracellular Ca^{2+} stores. In both cases specific Ca^{2+} channels mediate the transmembraneous Ca^{2+} fluxes.

Putney proposed for the first time that emptying of internal Ca^{2+} stores is linked to a so called store-operated Ca^{2+} influx from the extracellular space (Putney 1986). Currently, a variety of receptors have been found to couple intracellular Ca^{2+} release via the $InsP_3$ receptor to Ca^{2+} influx in excitable as well as non-excitable cells (Barrit 1999; Hofmann et al. 2000). Two major mechanisms are proposed to explain store-operated Ca^{2+} influx (Montell 2001; Putney and McKay 1999): first, a yet not identified Ca^{2+} influx factor is proposed which is thought to trigger Ca^{2+} influx from voltage-insensitive Ca^{2+} channels (Randriamampita and Tsien 1993; Rzigalinski et al. 1999). Secondly, Irvine proposed a conformational coupling between the inositol 1,4,5-triphosphate ($InsP_3$) receptors and store-operated Ca^{2+} channels analogous to the conformational and functional coupling between the ryanodine receptors and L-type Ca^{2+} channels in skeletal muscle (Irvine 1990). The store oper-

ated Ca^{2+} current is in part thought to be mediated by ion channels of the transient receptor potential-canonical (TRPC) family. TRPC channels have been found to couple to both intracellular Ca^{2+} channels, the $InsP_3$ receptor and the ryanodine receptor (Kiselyov et al. 1998, 2000; Rosado and Sage 2000). Human TRPC3 channels stably expressed in HEK293 cells were found to couple exclusively to either $InsP_3$ receptors or ryanodine receptors (Kiselyov et al. 2000).

A direct contact between the α_{1S}-subunit of the L-type Ca^{2+} channel and the skeletal muscle ryanodine receptor (RyR type 1) is a structural prerequisite for excitation-contraction (EC) coupling in skeletal muscle (Schneider 1994). Consequently, depolarisation is sufficient to trigger intracellular Ca^{2+} transients via the RyR type 1 in the absence of a detectable Ca^{2+} influx (O'Brian et al. 2002). The intracellular Ca^{2+} stores, namely the sarcoplasmic reticulum, have limited capacity for Ca^{2+} storage. Therefore, efficient refilling of these stores is essential for repeated cycles of muscle contraction and relaxation. The L-type Ca^{2+} channel has been proposed to compensate for this loss of Ca^{2+} but it is believed that Ca^{2+} channels which activate the RyR type 1 do not serve as Ca^{2+} conductors (O'Brian et al. 2002; Tanabe et al. 1990, 1991). That is why the voltage-insensitive Ca^{2+} influx may play a prominent role to supply the muscle cell with sufficient Ca^{2+} (Hopf et al. 1996a; Kurebayashi and Ogawa 2001).

Myotubes derived from human satellite cells are a prototypical system for investigation of RyR type 1 mediated Ca^{2+} transients. In the present work we have used differentiated human skeletal muscle cells as a native model system to investigate the linkage of the selective depletion of ryanodine sensitive Ca^{2+} pools to voltage insensitive Ca^{2+} influx. We used specific pharmacological tools to exclude the involvement of the phospholipase C products $InsP_3$ and diacylglycerol in this store operated Ca^{2+} influx. We could also identify a diacylglycerol dependent Ca^{2+} influx which was strictly dependent on protein kinase C activity.

Materials and methods

Materials. Sera and media for cell culture were obtained from PAA (Linz, Austria), trypsin-EDTA, glutamine, penicillin, streptomycin, gentamicin and amphotericin B were obtained from GibcoBRL (Vienna, Austria), xestospongin C, 1,6-bis (cyclohexyloximinocarbonylamino) hexane (RHC-80267), 1,2-Dioctanoyl-sn-glycerol (DOG), 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3- (1H-indol-3-yl)-maleimide (GF109203X), staurosporine and nifedipine from Calbiochem (San Diego, CA, USA), aprotinin from Bayer AG (Wuppertal, Germany), Pefabloc from Boehringer Mannheim (Mannheim, Germany), fibronectin from Collaborative Biomedical Products (Bedford, MA, USA). All other chemicals were from Sigma-Aldrich (Vienna, Austria).

Cell culture. The study conformed to the code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Ethics Committee of the AKH Wien (University Hospital of Vienna). Patients, who underwent the in vitro contracture test to verify susceptibility for malignant hyperthermia (MH) gave informed written consent to use waste material from their muscle biopsies (200–400 mg) for muscle cell culture. The present study was carried out with cells from eight individuals, found to be MH non susceptible. Human skeletal muscle cells were prepared and cultured according to the method of Brinkmeier et al. (1993) and grown in growth medium (GM) or differentiation medium (DM) according to Baroffio et al. (1993). GM contained Ham's F12 supplemented with 15% foetal calf serum 10 ng/ml EGF, 200 ng/ml insulin, 400 ng/ml dexamethasone, 0.5 mg/ml fetuin, 0.5 mg/ml BSA, 7 mM glucose, 4 mM L-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin, 2.5 µg/ml amphotericin B. DM contained: DMEM supplemented with 5% horse serum, and 4 mM L-glutamine, 100 ng/ml insulin, 0.1 µg/ml gentamicin.

Cells were grown at 37°C under 2.5% CO_2 close to confluency and reseeded on 25 mm glass cover slips coated with fibronectin for imaging experiments, or 75 cm^2 flasks for mass cell fluorescence photometry. Thereafter adherent cells were switched to DM and 5% CO_2 to obtain myotubes. Experiments were done within a period of 3 to 10 days after changing the culture medium from GM to DM.

Ca2+ imaging and fluorescence photometry. Ca^{2+} fluorescence imaging (Nikon Diaphot 300 fluorescence microscope) was carried out with myotubes incubated in 7 µM fura2/AM. Dye loading conditions were identical as previously described (Weigl et al. 2000). Fluorescence intensity was monitored at an emission wavelength of 510 nm with excitation by a monochromator at 340 and 380 nm (VisiTech, Sunderland, UK). Stored images were analysed using the QC 900 software package. Regions of interest were defined covering the whole visible area of a cell and the corresponding light intensity values were further processed with the Sigma Plot program (SPSS Inc., Erkrath, Germany). Background subtraction, ratioing and calculation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were done off-line. Calculation of $[Ca^{2+}]_i$ values was carried out according to the equation of Grynkiewicz et al. (1985). The parameters for the equation were obtained by a calibration procedure in which we used the penta-potassium salt of fura2 (5 µM) in a solution mimicking the intracellular milieu (Thomas and Delaville 1991). The K_D of Ca^{2+} for fura2 was assumed to be 224 nM. The fluorescence ratio in the absence of Ca^{2+} (R_{min}) gave 0.39 and was 10.64 in the presence of saturating concentrations of Ca^{2+} (R_{max}). The quotient (β) of the fluorescence values for Ca^{2+} bound and Ca^{2+} free dye at 380 nM was 10.75.

The quantification of the Ca^{2+} influx was obtained from subtracting basal Ca^{2+} concentrations after the drug application (caffeine or nifedipine) in the absence of extracellular Ca^{2+} from the peak Ca^{2+} concentration in the presence of extracellular Ca^{2+} (cf. arrow in Fig. 1B). Inhibition of the Ca^{2+} by La^{3+} was analysed in the same manner. Fluorescence-quench experiments were monitored in the presence of 500 µM $MnCl_2$ at an excitation wavelength of 359 nm (corresponding to the isosbestic point) and an emission wavelength of 510 nm.

Application of compounds to single cells was performed by a superfusion system with a 7-channel perfusion pipette (List-electronic, Darmstadt, Germany), driven by a valvebank (TSE, Bad Homburg, Germany) with solution exchange times of less than 500 ms. For experiments we used multinucleated cells only which showed a rise in $[Ca^{2+}]_i$ when challenged with Ca^{2+} free depolarisation solution (depolarisation solution HK: Tyrode's solution, but with 60 mM KCl and 80 mM NaCl). Based on the current model of skeletal muscle EC coupling, these cells fulfil essential criteria for the presence of intact functional coupling between the L-type Ca^{2+} channel and the RyR type 1. During measurements cells were constantly rinsed with fresh Tyrode's solution (concentrations in mM: NaCl 137, glucose 5.6, KCl 5.4, $NaHCO_3$ 2.2, $MgCl_2$ 1.1, NaH_2PO4, HEPES/Na 10, $CaCl_2$ 1.8, pH 7.4). Ca^{2+} free assay conditions were obtained by omitting Ca^{2+} and addition of 0.5 mM EGTA in Tyrode's solution. Application of compounds in the absence of Ca^{2+} was preceded for a few seconds by a short rinse of the cells with Ca^{2+} free solution to exclude an effect of Ca^{2+} deprivation.

In addition, fluorescence photometry was carried out with muscle cells in suspension using a Hitachi F-4500 photometer. Differentiated myotubes ($2–15\times10^6$ cells) were loaded with 4–10 µM fura2/AM (supplemented with 0.025% pluronic acid) for 35 and 60 min at 37°C in Tyrode's solution. The cells were washed twice

and finally resuspended in Tyrode's solution. Mn^{2+} influx was monitored at an excitation wavelength of 359 nm (corresponding to the isosbestic point evaluated at the photometer) and an emission wavelength of 510 nm in a manner analogous to the Ca^{2+} imaging experiments. Similar to single cell Ca^{2+} imaging differentiated human skeletal muscle cells in suspension responded to depolarisation with HK in the absence of extracellular Ca^{2+}, caffeine or nifedipine. Undifferentiated skeletal muscle cells did not show an elevation in $[Ca^{2+}]_i$ by the application of nifedipine which was in the line of our previous findings in single cell Ca^{2+} imaging (Weigl et al. 2000). Light sensitive compounds were kept in dark until use and exposed to light for measurements not longer than 2 min.

RT-PCR experiments. Total RNA was isolated from differentiated human skeletal muscle cells or rat hippocampal neurones using the RNeasy Mini-kit from Qiagen. Subsequently, total RNA was reverse transcribed into first-strand cDNA with the RT-PCR-kit from Takara using oligo-dT and random primers. Aliquots of the cDNA were employed as templates for PCR amplification with specific primers for human TRPC 1, 3 and 4 taken from Groschner et al. (1998) and for TRPC 5 and 6 from Garcia and Schilling (1997). In control experiments, PCR was carried out with the following templates: total RNA, total RNA treated with DNase I or in the absence of a template. All these experimental conditions did not result in the amplification of positive PCR products. As a positive control TRPC 5 primers were used under identical PCR conditions with rat hippocampal cDNA as a template leading to the amplification of a 340 bp PCR product of the predicted size. The products were resolved on a 1.5% agarose gel by electrophoresis. In addition the PCR products were sequenced and found to be identical to the respective human isoforms.

Miscellaneous procedures. All experiments were repeated at least three times. Data are given as mean ± SEM unless otherwise stated. Statistical analysis for multiple comparison was performed using ANOVA and a subsequent post hoc Scheffe's test. For single comparison Student's *t*-test was carried out to test the significance of differences; a value of $p<0.05$ was considered statistically significant.

Results

Store-operated Ca^{2+} influx in human skeletal muscle cells

Differentiated primary human skeletal muscle cells typically responded with a Ca^{2+} transient in the absence of extracellular Ca^{2+} when depolarised by high potassium concentrations (HK) (Fig. 1A). The formation of an intact triad, namely the apposition of the voltage-sensitive L-type Ca^{2+} channels in the plasma membrane and the RyR type 1 of the sarcoplasmic reticulum, is a prerequisite for such HK induced intracellular Ca^{2+} transients (Franzini-Armstrong 2000; Schneider 1994). In contrast, simple omission of extracellular Ca^{2+} in the bath solution had no effect on the fura2 fluorescence trace (Fig. 1A). Omission and re-addition of extracellular Ca^{2+} was not sufficient to trigger a Ca^{2+} release or Ca^{2+} influx (Fig. 1A). Caffeine which directly activates the RyR type 1, triggered intracellular Ca^{2+} transients although extracellular Ca^{2+} was not present (Fig. 1B) (Herrmann-Frank et al. 1999; Hohenegger et al. 1996; Klinger et al. 1999; Liu and Meissner 1997). Typically, after application of caffeine in the absence of extracellular Ca^{2+}, an increase in the $[Ca^{2+}]_i$ trace was detectable upon Ca^{2+} re-addition to the extracellular bath solution (indicated by an arrow in Fig. 1B). We compared the basal Ca^{2+} level in the absence of extracellular Ca^{2+} with the peak Ca^{2+} level in the presence of extracellular Ca^{2+} 15 s after the end of a 30 mM caffeine pulse. The intracellular Ca^{2+} concentration was augmented from 62.8±4.5 nM to 101.6±11.4 nM ($p<0.005$; $n=23$). This elevation in the Ca^{2+} signal could be completely suppressed by La^{3+} which was previously found to block store operated Ca^{2+} influx (Kwan et al. 1990; Okada et al. 1998; Robertson et al. 2000) (cf. Fig. 1B, D). The caffeine induced Ca^{2+} transient is strictly dependent on filled Ca^{2+} stores. Depletion of intracellular Ca^{2+} stores by thapsigargin, a blocker of the Ca^{2+} ATPase of the sarcoplasmic reticulum (Thastrup et al. 1994) completely prevented caffeine induced Ca^{2+} responses (Fig. 1C).

As already seen in Fig. 1B (indicated by an arrow), the tail of the caffeine induced Ca^{2+} transient was elevated upon switching from an extracellular solution free of Ca^{2+} (white bars) to a Ca^{2+} containing medium (grey bars). In thapsigargin treated cells this buffer change evoked the same phenomenon of Ca^{2+} influx (Fig. 1C), but was independent of the presence of caffeine. Again this store operated Ca^{2+} influx was completely blocked by 1 mM La^{3+} in a reversible manner and was only seen when Ca^{2+} was supplemented in the extracellular bath solution (grey bars in Fig. 1C). As depicted in Fig. 1, store operated Ca^{2+} influx was inhibited by La^{3+}, irrespective of whether store depletion was induced by caffeine (Fig. 1B, D) or thapsigargin (Fig. 1C). In both cases, upon wash-out of La^{3+} the inhibition was reversed.

It is yet not clear from the literature which voltage-insensitive Ca^{2+} influx channels are present in human skeletal muscle. Assuming that TRPC channels participate in Ca^{2+} influx it should be possible to detect their message using RT-PCR. On transcriptional level human TRPC1, TRPC3, TRPC4 and TRPC6 channels are present in human skeletal muscle cells (Fig. 2). Using a temperature gradient during the PCR reaction we controlled simultaneously for the specificity of the PCR products. At a very low primer annealing temperature of 42°C a non-specific band is visualised for TRPC 3 isoform which was vanished at higher temperatures. Nevertheless, all PCR products were purified from agarose gels and their identity was confirmed by sequencing. The primer pairs were chosen in order to obtain products that crossed intron-exon borders. This implies that the mRNA for TRPC 1, 3, 4 and 6 channels is correctly transcribed and spliced, and the observed products did not result from a contamination of genomic DNA.

Linkage of the ryanodine-sensitive Ca^{2+} pool to Ca^{2+} influx

In order to further confirm a link between the ryanodine-sensitive Ca^{2+} pool and store operated Ca^{2+} influx, additional experiments were carried out using the dihydropyridine, nifedipine. We recently demonstrated, that the

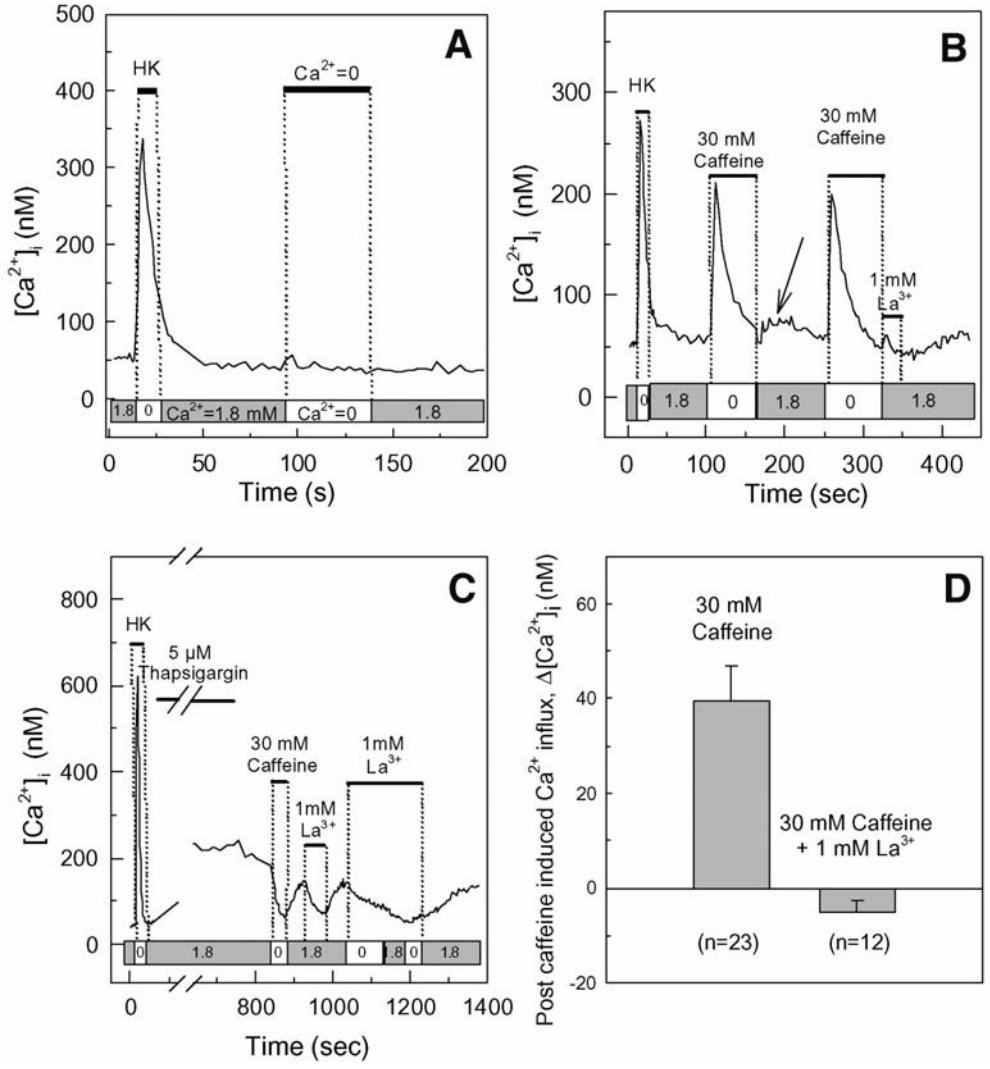

Fig. 1A–D Voltage-insensitive Ca^{2+} influx is present in human skeletal muscle cells. Differentiated human skeletal muscle cells loaded with fura2/AM were kept in Tyrode's solution for Ca^{2+} fluorescence imaging (*grey bars*; Ca^{2+}=1.8 mM) or challenged in the absence of Ca^{2+} (Tyrode's solution without Ca^{2+} but with 0.5 mM EGTA; *white bars*; Ca^{2+}=0). Multinucleated cells which responded to depolarisation solution (HK) in the absence of extracellular Ca^{2+} were considered to be differentiated skeletal muscle cells. **A** Switching from Ca^{2+} containing medium to Ca^{2+} free medium and back to Ca^{2+} containing extracellular solution did not alter intracellular Ca^{2+} concentrations. **B** $[Ca^{2+}]_i$ was monitored upon application of depolarisation solution (HK), 30 mM caffeine and 1 mM La^{3+}. Caffeine-induced Ca^{2+}-release was observed in the absence of extracellular Ca^{2+} (*white bars*) and was followed by a Ca^{2+} influx (*arrow*). Subsequent Ca^{2+} influx upon extracellular Ca^{2+} re-addition (*grey bars*) is inhibited by La^{3+}. **C** After depolarisation of a skeletal muscle cell with HK in the absence of extracellular Ca^{2+} the cells were treated with 5 µM thapsigargin for the indicated time and subsequently exposed to 30 mM caffeine and 1 mM $LaCl_3$ as indicated. **D** After the addition of 30 mM caffeine in the absence of extracellular Ca^{2+} subsequent Ca^{2+} influx (as indicated in panel **B** by an *arrow*) was quantified. The increment in intracellular Ca^{2+} concentration was measured 15 s after ceasing caffeine application and expressed as $\Delta[Ca^{2+}]_i$. Ca^{2+} influx was completely suppressed by 1 mM La^{3+} (Student's *t*-test, $p<0.0005$). The *bars* indicate the mean and SD; the number of experiments (*n*) is given in parenthesis

L-type Ca^{2+} channel antagonist nifedipine is able to induce Ca^{2+} release from ryanodine-sensitive Ca^{2+} pools in human skeletal muscle cells, although under these conditions the L-type Ca^{2+} channel is inhibited and no Ca^{2+} influx can occur via this channel (Weigl et al. 2000). Therefore nifedipine was suitable as a second compound to selectively release Ca^{2+} from ryanodine-sensitive Ca^{2+} pools but now under conditions where we can exclude Ca^{2+} influx via the L-type Ca^{2+} channel (dihydropyridine receptor). Again, when intracellular Ca^{2+} stores in human skeletal muscle cells were depleted with 1 µM thapsigargin, similar to the conditions given in Fig. 1C, the basal intracellular Ca^{2+} concentration was elevated. Under such conditions the simple omission of extracellular Ca^{2+} (Fig. 3, white bars) followed by re-addition of Ca^{2+} (Fig. 3, grey bars) led to a Ca^{2+} influx. Nifedipine was not able to induce a Ca^{2+} transient in thapsigargin treated cells; independent whether extracellular Ca^{2+} was absent or present (Fig. 3).

Conversely, in differentiated muscle cells which were not exposed to thapsigargin, 10 µM nifedipine clearly evoked a Ca^{2+} transient (Fig. 4B). The Ca^{2+} transient induced by nifedipine was broader compared to caffeine

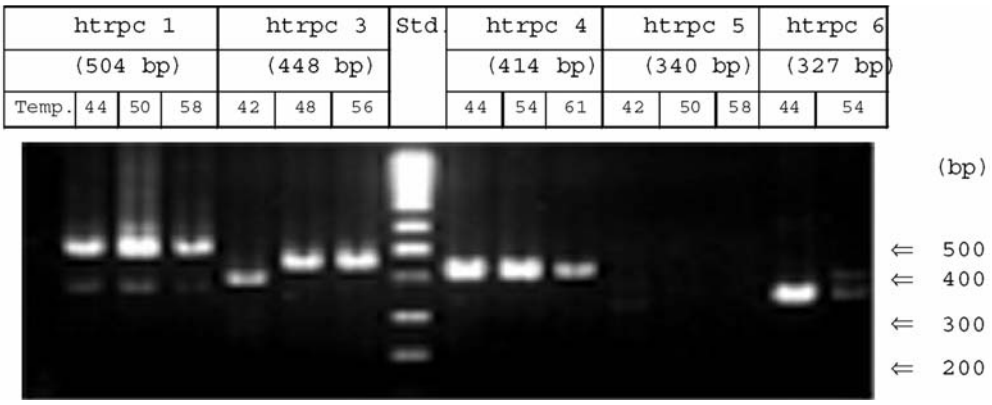

Fig. 2 RT-PCR products for specific human TRPC channel isoforms. PCR products from RT-PCR with specific primers for the given isoforms of human TRPC channels were resolved on a 1.5% agarose gel and visualised by ethidium bromide. The expected size of PCR products are given in parenthesis and the different annealing temperatures indicated below. The melting temperatures calculated from the forward and reverse primer sequence were 50°C and 53°C for TRPC 1, 48°C and 44°C for TRPC 3, 60°C and 54°C for TRPC 4, 48°C and 58°C for TRPC 5 and 48°C and 59°C for TRPC 6, respectively. The molecular mass of the 100 bp-DNA ladder standard (*std.*) is indicated by the *arrows*

and seems to be composed of a second sustained Ca^{2+} release phase (cf. Fig. 1B with Fig. 4B). In the presence of extracellular Ca^{2+} the transient lasted for the duration of the drug application and was fully reversible upon washout (Fig. 4B). This is consistent with our previous findings (Weigl et al. 2000). Simultaneous addition of 100 µM Cd^{2+}, an inhibitor of voltage sensitive and insensitive Ca^{2+} channels (Parekh and Penner 1997), did not prevent the initiation of the nifedipine induced Ca^{2+} transient. However, it turned the sustained shape of the nifedipine induced Ca^{2+} elevation into a sharp Ca^{2+} transient (Fig. 4A). We have quantified this effect in the absence and presence of Cd^{2+} by comparing the peak amplitude of the nifedipine induced Ca^{2+} transient with the Ca^{2+} concentration after 50 s of drug application. The intracellular Ca^{2+} concentration at this time point was 92.3±3.0% (n=5) of the peak amplitude in the absence of Cd^{2+}. In the presence of 100 µM Cd^{2+} the intracellular Ca^{2+} concentration was reduced to 43.9±7.0% (n=5; p=0.0007) of the peak amplitude of the nifedipine induced Ca^{2+} transient. This confirms our previous finding that binding of nifedipine to the dihydropyridine binding site of the L-type Ca^{2+} channel is sufficient to trigger the RyR type 1 opening (Weigl et al. 2000). Ca^{2+} influx initiated by the application of 10 mM caffeine was also inhibited by 100 µM Cd^{2+}, while the caffeine induced Ca^{2+} release was unaffected (data not shown).

Similar to caffeine, nifedipine significantly triggered a Ca^{2+} influx (control: 83.8±5.0 nM; peak $[Ca^{2+}]_i$ after 10 µM nifedipine 107.0±6.8 nM; p=0.01; n=12) (Fig. 4B, C). Again lanthanum, which inhibited caffeine induced Ca^{2+} influx (Fig. 1D), significantly suppressed nifedipine induced Ca^{2+} influx (Fig. 4B, C). The sensitivity of the Ca^{2+} influx to Cd^{2+} and La^{3+} provided evidence for participation of TRPC channels on this phenomenon (Parekh and Penner 1997).

Visualisation of Ca^{2+} influx by Mn^{2+} induced quenching of fluorescence

A common method to visualise Ca^{2+} influx is to make use of the isosbestic wavelength of fura2. Upon excitation at 359 nm, fura2 does not respond to changes in $[Ca^{2+}]_i$. However, a Mn^{2+} influx through plasmalemmal Ca^{2+} channels quenches the intracellular fluorescence signal which is therefore proportional to the Mn^{2+} influx (Hopf et al. 1996a; Kass et al. 1994; Kurebayashi and Ogawa 2001; Xu et al. 1994). Using the Ca^{2+} imaging technique, simultaneous recordings of the fluorescence signal of fura2 loaded differentiated skeletal muscle cells excited at 340 nm, 359 nm and 380 nm are presented in Fig. 5. Usually, in the presence of HK or caffeine the muscle cells contracted which caused an artefact in the 359 nm fluorescence trace. However, upon addition of 500 µM Mn^{2+}, a

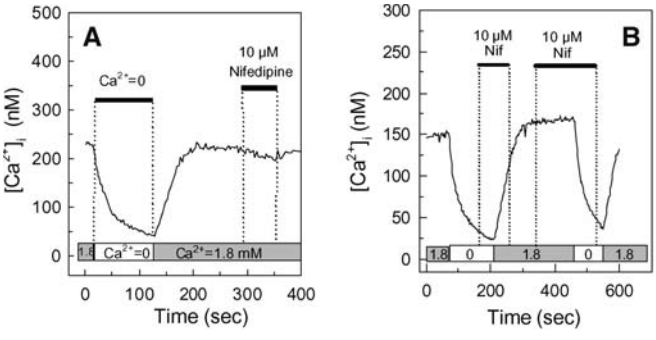

Fig. 3A, B Nifedipine induced intracellular Ca^{2+} transients and Ca^{2+} influx. Fura2/AM loaded differentiated human skeletal muscle cells which reacted to HK with a $[Ca^{2+}]_i$ transient (not shown) were incubated in 1 µM thapsigargin for 15 min. **A** Sequentially, the cells were exposed to Ca^{2+} containing Tyrode's solution (*grey bars*) and Ca^{2+} free Tyrode's solution (*white bar*). Under conditions of depleted intracellular Ca^{2+} stores nifedipine was not able to trigger a Ca^{2+} transient in the presence of extracellular Ca^{2+}. **B** Under the same conditions of thapsigargin treatment myotubes were subjected to 10 µM nifedipine (*Nif*) either in the presence or absence of extracellular Ca^{2+}. Under conditions of depleted Ca^{2+} stores, the change in $[Ca^{2+}]_i$ is solely dependent on the extracellular $[Ca^{2+}]$ and not on the presence of nifedipine

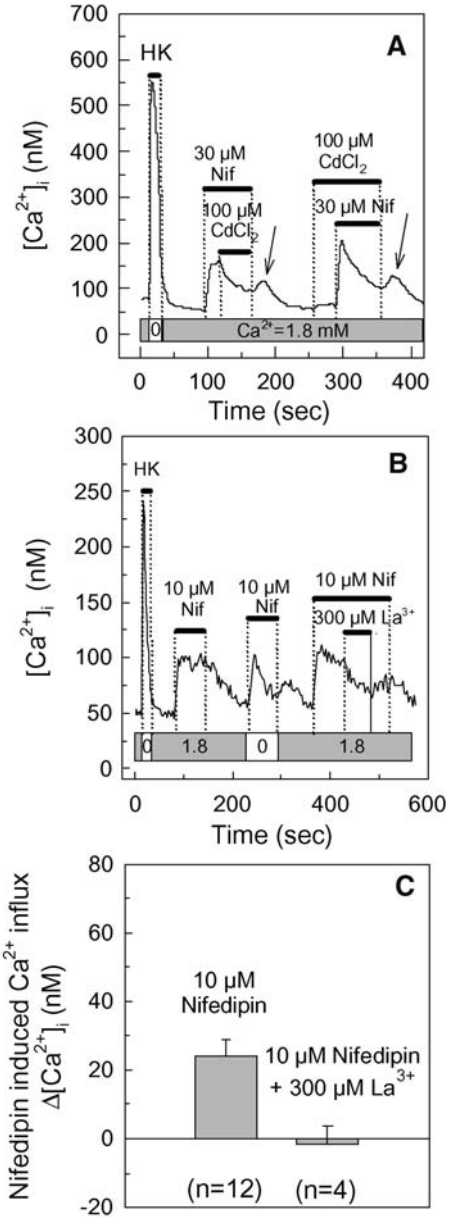

Fig. 4A–C Nifedipine induced Ca^{2+} influx is inhibited by Cd^{2+} and La^{3+}. **A** Nifedipine induced Ca^{2+} release in fura2 loaded differentiated skeletal muscle cells was independent of whether 100 µM $CdCl_2$ was present prior to the nifedipine addition or added afterwards. The *arrows* indicate a Ca^{2+} elevation. **B** Similar to panel **A**. A differentiated skeletal muscle cell loaded with fura2 was repeatedly exposed to 10 µM nifedipine (*Nif*). The cell was sequentially exposed to Ca^{2+} containing Tyrode's solution (*grey bars*; 1.8 mM Ca^{2+}) or Ca^{2+} free Tyrode's solution (*white bars*; zero Ca^{2+}). The addition of 300 µM La^{3+} blocked the Ca^{2+} influx component of the nifedipine-induced Ca^{2+} transient similar to Cd^{2+}. **C** The Ca^{2+} influx induced by 10 µM nifedipine was quantified similar to Fig. 1D. The Ca^{2+} influx was significantly inhibited in the presence of 300 µM La^{3+} (Student's *t*-test, p=0.01). The *bars* indicate the mean and SD; the number of experiments (n) is given in parenthesis

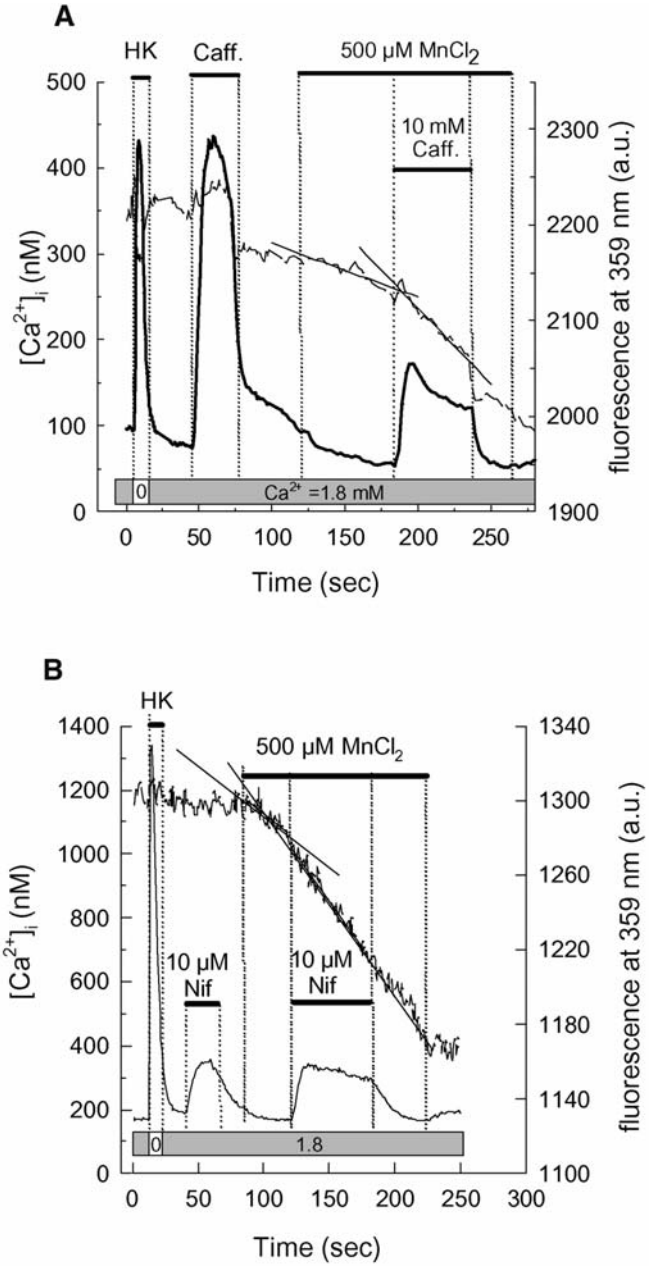

Fig. 5A, B Caffeine and nifedipine trigger Mn^{2+} influx in skeletal muscle cells. **A** Fluorescence of a fura2 loaded myotube was monitored simultaneously at an excitation wavelength of 340 nm and 380 nm (*thick line*) and at its isosbestic point (excitation wavelength 359 nm; *dotted line*). *Horizontal lines* indicate the drug application. Basal Mn^{2+} influx was triggered by the addition of 10 mM caffeine (*Caff.*) and resulted in an acceleration of fluorescence quenching. The regression lines of the isosbestic fluorescence trace before and after caffeine addition are given as overlays. **B** Under conditions similar to panel **A** Mn^{2+} influx was also stimulated by 10 µM nifedipine (*Nif*). The Ca^{2+} content of the extracellular solution is given by *white* (zero Ca^{2+}) and *grey* (1.8 mM Ca^{2+}) *bars*

basal Mn^{2+} influx was observed which was clearly accelerated by the co-application of 10 mM caffeine or 10 µM nifedipine (Fig. 5A, B, respectively). The slopes were calculated by linear regressions and used as estimates of the Mn^{2+} influx rate. The basal rate of Mn^{2+} influx (0.6±0.06 a.u./s; mean ± SEM, n=8) was significantly increased two- (1.05±0.13 a.u./s; mean ± SEM, p<0.05; n=3) and three-fold (1.94±0.31 a.u./s; mean ± SEM, p<0.005; n=4) in the presence of nifedipine and caffeine, respectively.

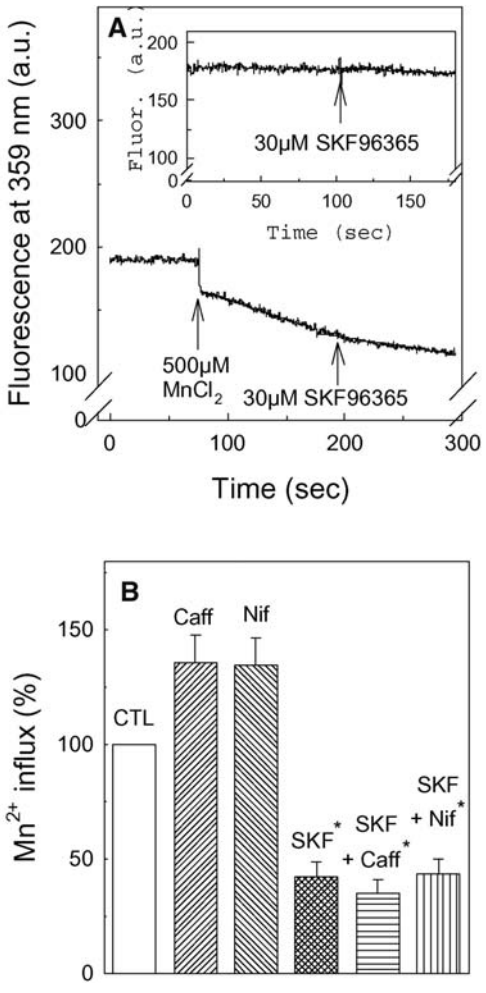

Fig. 6A, B SKF96365 inhibits Mn^{2+} influx in human skeletal muscle cells. **A** Fura2 loaded differentiated human skeletal muscle cells (2–4×10^5 cells) were kept in Tyrode's solution and used for fluorescence photometry at the isosbestic point of fura2 to monitor the fluorescence quenching by Mn^{2+}. The application of 500 µM $MnCl_2$ and 30 µM SKF96365 is indicated by the *arrows*. 30 µM SKF96365 per se had no effect on the fluorescence signal as given in the inset of panel **A**. **B** The percentage of the activation and inhibition of the Mn^{2+} influx was employed under conditions similar to panel **A**. The Mn^{2+} influx was stimulated by 10 mM caffeine (*Caff*; *n*=12), 10 µM nifedipine (*Nif*; *n*=9) and inhibited by 30 µM SKF96365 in the absence (*SKF*; *n*=10) or presence of 10 mM caffeine (*SKF*/*Caff*; *n*=7) and 10 µM nifedipine (*SKF*/*Nif*; *n*=10). Data in panel **B** were normalised by setting the basal Mn^{2+} influx in the absence of any drug 100%. The mean percentage of the Mn^{2+} influx is depicted and the standard deviation indicated by *error bars*. Single factor analysis of variance (ANOVA) of the data and subsequent Scheffe's post hoc comparison resulted in significance for all drug applications considering a p value <0.05

Ca^{2+} influx in skeletal muscle cell suspensions

The results for the fura2 loaded skeletal muscle cells in cell suspension were similar compared to the results presented on single cell level in Fig. 5. Mn^{2+} was added to the extracellular bath solution which resulted in a rapid drop in fluorescence intensity correlating to the quenching of extracellular fura2 (Fig. 6A). This initial step in fluorescence was followed by a basal Mn^{2+} influx comparable to the isosbestic fluorescence traces in Fig. 5. SKF96365, an inhibitor of voltage-insensitive Ca^{2+} influx (Okada et al. 1998; Zhu et al. 1998), per se had no effect on the isosbestic fluorescence (insert in Fig. 6A; Ex: 359 nm, Em: 510 nm). However, SKF96365 inhibited Mn^{2+} influx immediately (Fig. 6A). The fluorescence traces were again analysed by linear regressions and expressed as arbitrary fluorescence units (a.u.) per time and normalised to the basal Mn^{2+} influx in the absence of any drug. The initial quenching of free fura2 was not introduced into the calculation. Analogous to experiments on single cell level, basal Mn^{2+} influx was further enhanced by intracellular Ca^{2+} store depletion due to application of nifedipine or caffeine (Fig. 6B). SKF96365 blocked the Mn^{2+} influx irrespective of either nifedipine or caffeine was used to elicit it. Although SKF96365, La^{3+} and Cd^{2+} have been described to be not specific for store operated Ca^{2+} influx channels they have been extensively used and shown to block some TRPC channels (Parekh and Penner 1997). In conclusion of our observations, the Ca^{2+} influx and Mn^{2+} influx was abolished by SKF96365, La^{3+} and Cd^{2+} in human skeletal muscle cells, not only on single cell level but also in mass suspension.

Ca^{2+} influx by the depletion of ryanodine-sensitive Ca^{2+} pools is independent of phospholipase C activity

Hitherto, it is clear that depletion of the ryanodine-sensitive Ca^{2+} pool suffices to trigger Ca^{2+} influx from the extracellular compartment. 1,2-Dioctanoyl-sn-glycerol (DOG), a cell permeable analogue of diacylglycerol should activate TRPC3 and TRPC6 channels (Hofmann et al. 1999), which were present on mRNA level in skeletal muscle cells (Fig. 2). DOG was dissolved in DMSO which did not exceed a final concentration of 0.1% (v/v), a concentration which was without effects on $[Ca^{2+}]_i$ (Weigl et al. 2000). Furthermore, it should be expected that an inhibitor of the diacylglycerol lipase, RHC-80267, further amplifies this Mn^{2+} influx. In fact, basal Mn^{2+} influx in the presence of 100 µM DOG was increased to 120.1±4.4% (*n*=7; mean ± SD) (Fig. 7). When 80 µM RHC-80267 was applied and successively followed by the addition of 100 µM DOG the increment in Mn^{2+} influx reached 144.4±15% (*n*=4; mean ± SD). By comparison, Mn^{2+} influx evoked by 10 mM caffeine or 10 µM nifedipine was not altered by the co-application of the diacylglycerol lipase inhibitor (Fig. 7B). The sole addition of 80 µM RHC-80267 had no detectable effect on Mn^{2+} influx.

The DOG induced acceleration in Mn^{2+} influx may be due to an activation of a protein kinase C. Therefore, Mn^{2+} influx was investigated in the absence and presence of inhibitors of the protein kinase C. GF109203X and staurosporine, two protein kinase C inhibitors, had no influence on basal Mn^{2+} influx (Table 1). The addition of DOG or the combination of DOG plus RHC-80267 augmented basal Mn^{2+} influx significantly. The same was true

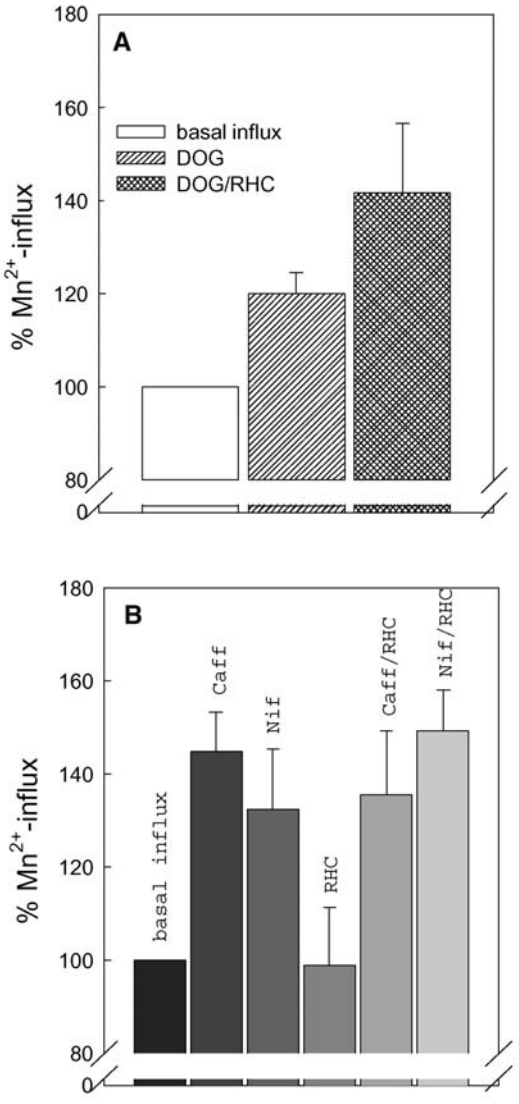

Fig. 7A, B Diacylglycerol does not participate in caffeine or nifedipine stimulated Mn^{2+} influx. **A** Under conditions given in the legend of Fig. 6A the fluorescence signal of fura2 at the isosbestic point was determined after the application of 500 µM $MnCl_2$. The effect on basal Mn^{2+} influx (set 100%; *open bar*, *n*=11) was compared to the changes in steepness of the Mn^{2+} influx due to subsequent addition of 100 µM DOG (*DOG*; *hatched bar*, *n*=7) and 100 µM DOG with 80 µM RHC-80267 (*DOG/RHC*; *cross hatched bar*, *n*=4). **B** Under identical conditions as in panel **A** the effect of RHC-80267 on Mn^{2+} influx was investigated. Basal Mn^{2+} influx was set 100% (*open bar*; *n*=15) and stimulated either by 10 mM caffeine (*Caff*; *n*=4), 10 µM nifedipine (*Nif*; *n*=4) or 80 µM RHC-80267 (*RHC*; *n*=4). Simultaneously, 500 µM $MnCl_2$ plus 80 µM RHC-80267 was applied and with a delay of 2 min 10 mM caffeine (*Caff/RHC*; *n*=5) or 10 µM nifedipine (*Nif/RHC*; *n*=5) were added. The *bars* indicate the mean percentage of the Mn^{2+} influx determined by linear regression and the standard error of mean is indicated by *error bars*

for caffeine and nifedipine. Interestingly, the protein kinase C inhibitors had no effect on the increment of DOG induced Mn^{2+} influx. In contrast, the Mn^{2+} influx evoked by DOG plus RHC-80267 was significantly inhibited by 65%. This finding may support the conjecture that a protein kinase C is involved in DOG plus RHC-80267 mediated Mn^{2+} influx. Data for Mn^{2+} influx triggered by caffeine or nifedipine were controversial in the presence of protein kinase C inhibitors. GF109203X inhibited caffeine triggered Mn^{2+} influx slightly (17%). However, in all other cases protein kinase C inhibitors did not alter caffeine or nifedipine induced Mn^{2+} influx. In staurosporine pre-treated cells, nifedipine induced Mn^{2+} influx was significantly augmented (44% stimulation; $p<0.05$). Overall, the acceleration in Mn^{2+} influx was much more pronounced when skeletal muscle cells were allowed to differentiate for 10 days (Table 1) compared to 4 days in experiments shown in Figs. 6 and 7. Taken together, the data presented so far demonstrate that caffeine and nifedipine accelerate Mn^{2+} influx independently of the presence of the second messenger diacylglycerol (Fig. 7). While DOG plus RHC-80267 induced Mn^{2+} influx is completely blocked by protein kinase C inhibitors, the participation of protein kinase C in caffeine or nifedipine induced Mn^{2+} influx is unlikely (Fig. 7, Table 1).

Although improbable, formally it cannot be ruled out that caffeine or nifedipine induced Mn^{2+} influx is dependent on the activation of the $InsP_3$ receptor. We have used the phospholipase C inhibitor U73122 to block the synthesis of $InsP_3$ (Berridge et al. 1998). Concentrations of 5–25 µM U73122 were not able to inhibit Mn^{2+} influx triggered by caffeine or nifedipine (data not shown). As already mentioned transactivation of the $InsP_3$ receptor by elevated $[Ca^{2+}]_i$ represents another possibility to explain Ca^{2+} influx after depletion of ryanodine sensitive Ca^{2+} stores. In order to address this question, we have exposed differentiated muscle cells to the cell permeable $InsP_3$ receptor inhibitor xestospongin C. Surprisingly, the resting Ca^{2+} concentration under xestospongin C treatment was augmented from 59.5±13.8 a.u. (*n*=17) to 216±25.6 a.u. (mean ± SD; *n*=21; $p<0.001$). This observation may be due to unspecific effects of xestospongin C (Bootmann et al. 2002). In particular, inhibition of the Ca^{2+} ATPase of the sarcoplasmic reticulum is a likely explanation for the increase in $[Ca^{2+}]_i$ as this is observed also for other Ca^{2+} ATPase inhibitors like thapsigargin (De Smet et al. 1999). Nevertheless, in the presence of 10 µM xestospongin C, caffeine or nifedipine induced Mn^{2+} influx was not altered in differentiated human skeletal muscle cells (data not shown). In conclusion, neither the phospholipase C products $InsP_3$ and diacylglycerol nor protein kinase C contributed to a Mn^{2+} influx induced by selective depletion of ryanodine-sensitive Ca^{2+} pools by caffeine or nifedipine.

Discussion

In the present study we investigated voltage-insensitive Ca^{2+} influx by selective depletion of the ryanodine-sensitive Ca^{2+} pool in differentiated human skeletal muscle cells. The mechanism of activation of this Ca^{2+} influx did not involve phospholipase C, protein kinase C or the $InsP_3$ receptor. These conclusions are based on the following observations:

Table 1 Diacylglycerol dependent Mn^{2+} influx but not caffeine or nifedipine induced Mn^{2+} influx is dependent on protein kinase C. Fura2 loaded differentiated skeletal muscle cells ($5–8\times10^5$) were exposed to Tyrode's solution in the absence (control) or presence of the protein kinase C inhibitors GF109203X or staurosporine. After 2 min of incubation with the PKC inhibitors 500 µM $MnCl_2$ (basal Mn^{2+} influx) or 500 µM $MnCl_2$ plus 100 µM DOG, 500 µM $MnCl_2$ plus 100 µM DOG and 80 µM RHC-80267 (RHC), 500 µM $MnCl_2$ plus 10 mM caffeine or 500 µM $MnCl_2$ plus 10 µM nifedipine was added. *Asterisks* and *crosses* indicate level of significance obtained from multiple comparison analysis with ANOVA and post hoc Scheffe's test

Mn^{2+} influx	Control, a.u./min, mean ± SEM (*n*)	+ 1 µM GF109203X, a.u./min, mean ± SEM (*n*)	+ 500 nM staurosporine a.u./min, mean ± SEM (*n*)
Basal Mn^{2+} influx	6.56±0.77 (13)	10.90±0.39 (5)	8.42±1.14 (13)
Plus DOG	13.65±0.98 (4)*	13.57±0.91 (6)	14.14±3.18 (6)
Plus DOG+RHC	32.55±3.46 (8)****	11.37±1.22 (13)$^{++++}$	11.36±1.96 (11)$^{++++}$
10 mM caffeine	27.87±6.41 (5)*	20.30±2.48 (11)	32.46±8.56 (6)
10 µM nifedipine	26.61±2.17 (9)***	35.15±5.51 (4)	38.34±4.77 (6)$^{+}$

$^{*}p<0.05$, $^{***}p<0.0001$, $^{****}p<0.00001$ vs. basal Mn^{2+} influx; $^{+}p<0.05$ vs. nifedipine; $^{++++}p<0.0001$ vs. DOG plus RHC-80267

1. Ca^{2+} influx occurs if the intracellular Ca^{2+} release is evoked by caffeine. While unlikely, it is not possible to formally exclude Ca^{2+} influx through L type Ca^{2+} channels (Figs. 1, 5)
2. We therefore used nifedipine to release Ca^{2+} via the RyR type 1. Although the L type Ca^{2+} channel is blocked under these conditions (Weigl et al. 2000), nifedipine induced Ca^{2+} influx (Figs. 4, 5)
3. Neither caffeine nor nifedipine were able to induce Ca^{2+} transients in thapsigargin treated skeletal muscle cells, which highlights the fact that both compounds trigger Ca^{2+} release and that this stimulus is the signal for Ca^{2+} influx (Figs. 1, 3)
4. Irrespective of whether Ca^{2+} influx was provoked by caffeine or nifedipine, it was not altered under conditions where the phospholipase C, protein kinase C or $InsP_3$ receptor were inhibited. Nevertheless, in staurosporine treated cells nifedipine induced Mn^{2+} influx was significantly enhanced (Table 1). This observation is unclear at the moment
5. Furthermore, the application of the diacylglycerol lipase inhibitor, RHC-80267, did not have an effect on caffeine or nifedipine induced Ca^{2+} influx (Fig. 7). These data allow the assumption that the observed Ca^{2+} influx due to nifedipine or caffeine is independent of products of the phospholipase C pathway, $InsP_3$ and diacylglycerol

In skeletal muscle for the first time Ca^{2+} influx through Ca^{2+} leak channels was shown by Hopf et al. (1996a). In mouse myotubes Ca^{2+} influx was provoked by the reversible Ca^{2+} ATPase inhibitor, cyclopiazonic acid. Similar to our experiments this Ca^{2+} influx was visualised by Mn^{2+} quench experiments and sensitive to La^{3+}. We also found store operated Ca^{2+} influx when the Ca^{2+} ATPase of the sarcoplasmic reticulum was blocked by thapsigargin (Figs. 1C, 3). This mechanism was also conserved in isolated skeletal muscle fibres where Kurebayashi and Ogawa (2001) detected Mn^{2+} influx when Ca^{2+} stores were depleted by Ca^{2+} ATPase inhibitors.

Recently, we found that the dihydropyridines nifedipine and nitrendipine increase the intracellular Ca^{2+} concentration with an EC_{50} of about 0.6 and 1.2 µM, respectively. This rise in $[Ca^{2+}]_i$ is due to activation of the RyR type 1 and subsequent entry of Ca^{2+} (Weigl et al. 2000). Hopf et al. (1996b) also found activation of Ca^{2+} leak channels by high concentrations of nifedipine (50 µM) in mouse myotubes and attributed this effect to direct activation of leak channels by this dihydropyridine. However, we previously showed that this rise in $[Ca^{2+}]_i$ is dependent on the intact EC-coupling mechanism and can be prevented e.g. by depolarisation of the cells to –30 mV which involves inactivation of the DHP receptor. In depolarised skeletal muscle cells nifedipine never induces Ca^{2+} release or Ca^{2+} influx (Weigl et al. 2000). Therefore we conclude that the Ca^{2+} influx triggered by nifedipine depends on intact EC-coupling and is not due to a direct activation of leak channels as proposed by Hopf at al. (1996b). The fact that Ca^{2+} influx occurs in the presence of 10 µM nifedipine which already blocks DHP receptors at nanomolar concentrations implies that this Ca^{2+} influx can not occur via DHP receptors and shows that there exists a mechanism which is able to replenish emptied ryanodine sensitive Ca^{2+} stores independently of the DHP receptor opening.

Candidates for ion channels which mediate voltage independent Ca^{2+} influx are the TRPC channels. Correctly spliced RT-PCR products were found for TRPC1, TRPC3, TRPC4 and TRPC6 channels in human myotubes (Fig. 2). The type 5 isoform of TRPC channels is expressed exclusively in the brain and was positive in the hippocampus (data not shown); accordingly, in myotubes we did not detect the mRNA of TRPC5 channel (Fig. 2; Philipp et al. 1998; Okada et al. 1998). Depending on the cell system TRPC3 and TRPC6 channels were either proposed to be store operated Ca^{2+} channels (Boulay et al. 1997; Mizuno et al. 1999) or activated via PLC linked receptors (Hofmann et al. 1999; Zitt et al. 1997). The direct activation of TRPC3 and TRPC6 channels by diacylglycerol was first described by Hofmann and co-workers (Hofmann et al. 1999). In line with these findings we also observed stimulation of Mn^{2+} influx by the cell permeable diacylglycerol analogue, DOG (Fig. 7A, Table 1). DOG was apparently metabolised rather rapidly, because co-application of the diacylglycerol lipase inhibitor, RHC-80267, clearly further accelerated the Mn^{2+} influx in skeletal muscle cells

(Fig. 7A, Table 1). Interestingly, the protein kinase C inhibitors, GF109203X and staurosporine, completely suppressed DOG plus RHC-80267 induced Mn^{2+} influx. In contrast, we failed to elucidate a participation of protein kinase C on DOG induced Mn^{2+} influx. Possibly, inter-assay variability of the preparation of human skeletal muscle cells and the small amplitude of the DOG induced increment in Mn^{2+} influx may explain this observation. However, when caffeine or nifedipine were administrated simultaneously with RHC-80267 the Mn^{2+} influx was not altered, indicating that protein kinase C is not involved in this Mn^{2+} influx. It is worth to mention that a member of the superfamily of TRP channels, the TRPM7 channel has been identified to contain a huge phospholipase C-interacting kinase at the COOH-terminal end which is functionally linked to conductivity of the ion channel (Runnels et al. 2001).

In conclusion, these data show that in skeletal muscle at least two independent Ca^{2+} influx mechanisms exist which can be separated pharmacologically. The depletion of ryanodine-sensitive Ca^{2+} stores is coupled to Ca^{2+} influx independent of phospholipase C, protein kinase C and the $InsP_3$ receptor. However, a phospholipase C dependent and protein kinase C dependent Ca^{2+} influx pathway obviously exists in human myotubes but is not involved in Ca^{2+} influx due to emptying of ryanodine-sensitive Ca^{2+} stores. We favour a model where the emptying of ryanodine-sensitive Ca^{2+} stores is coupled more directly to Ca^{2+} influx. The observation by Kiselyov et al. (2000) provides biochemical and functional evidence that ion channel tandems (TRPC3 channel/$InsP_3$ receptor and TRPC3 channel/ryanodine receptor) cluster and segregate in plasma membrane microdomains. Co-localisation of these ion channel complexes in the skeletal muscle can account for a signalling cascade that is triggered in parallel to EC-coupling. In a recent publication the synaptophysin-family-related protein, mitsugumin 29 was attributed to guide the ryanodine receptor to store operated Ca^{2+} channels (Pan et al. 2002). Store operated Ca^{2+} influx was inhibited by SKF96365> 90% and strictly dependent on the presence of the ryanodine receptor type 1 which is consistent with our findings (Fig. 6). In myotubes deficient in mitsugumin 29, store operated Ca^{2+} influx was virtually absent (Pan et al. 2002).

Voltage-independent Ca^{2+} influx plays a crucial role in the amplification, prolongation and modification of intracellular Ca^{2+} transients for example in T-lymphocytes (Alberola et al. 1997; Berridge 1997; Guse 1998). It is attractive to speculate that the implications of analogous mechanisms operate in skeletal muscle EC-coupling and are of potential relevance in the physiology and pathophysiology of skeletal muscle (Hopf et al. 1996b).

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