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Phosphorylation and IGF-1-mediated dephosphorylation pathways control the activity and the pharmacological properties of skeletal muscle chloride channels

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1 In the present study we tested the hypothesis that insulin-like growth factor-1 (IGF-1) modulates resting chloride conductance (G_{Cl}) of rat skeletal muscle by activating a phosphatase and that the chloride channel, based on the activity of phosphorylating-dephosphorylating pathways, has different sensitivity to specific ligands, such as the enantiomers of 2-(p-chlorophenoxy) propionic acid (CPP).

2 For this purpose G_{Cl} in EDL muscle isolated from adult rat was first lowered by treatment with 5 nM 4-β-phorbol 12,13 dibutyrate (4-β-PDB), presumably activating protein kinase C (PKC). The effects of IGF-1 and of the enantiomers of CPP on G_{Cl} were then tested.

3 IGF-1 (3.3 nM) had no effect of G_{Cl} on EDL muscle fibres in normal physiological solution, whereas it completely counteracted the 30% decrease of G_{Cl} induced by 4-β-PDB. No effects of IGF-1 were observed on G_{Cl} lowered by the phosphatase inhibitor okadaic acid (0.25 μM).

4 Ceramide, reported to activate on okadaic acid-sensitive phosphatase, mimicked the effects of IGF-1. In fact, N-acetyl-sphingosine (2.5–5 μM), not very effective in control conditions, increased the G_{Cl} lowered by the phorbol ester, but not the G_{Cl} lowered by okadaic acid.

5 In the presence of 4-β-PDB, G_{Cl} was differently affected by the enantiomers of CPP. The S(−)-CPP was remarkably less potent in producing the concentration-dependent reduction of G_{Cl}, whereas the R(+)-CPP caused an increase of G_{Cl} at all the concentrations tested.

6 In conclusion, the PKC-induced lowering of G_{Cl} is counteracted by IGF-1 through an okadaic acid sensitive phosphatase, and this effect can have therapeutic relevance in situations characterized by excessive channel phosphorylation. In turn the phosphorylation state of the channel can modulate the effects and the therapeutic potential of direct channel ligands.

Keywords: Chloride channels; skeletal muscle; insulin-like growth factor-1; phorbol esters; ceramide; phosphorylation; dephosphorylation; channel ligands

Introduction

A large chloride conductance (G_{Cl}) controls the electrical stability of resting sarcolemma of fast-twitch muscle fibres. This is confirmed by the spontaneous high frequency discharges of action potentials observed in myotonic muscle. In such muscle G_{Cl} is low for mutations of the gene coding for ClC-1, the putative muscle chloride channel (Pusch & Jentsch, 1994; Lehmann-Horn & Rüdel, 1996). Single-channel patch clamp studies fail to survey the channels that could account for the large G_{Cl} in healthy native muscle fibres. This is probably due to a small unitary channel conductance, as inferred from studies on CLC-1 expressed in heterologous cells (Pusch *et al*., 1994; Fahlke *et al*., 1997) and/or to a channel localization in the transverse tubules, inaccessible to the patch pipette (Chua & Betz, 1991). The lack of specific antibodies against ClC-1 has so far prevented biochemical clarification of these points.

Most of the information about the function and the sensitivity to both drugs and biochemical pathways of the native chloride channels is still obtained by macroscopic recordings of muscle G_{Cl}. By this approach we showed that G_{Cl} of rat skeletal muscle is strongly biochemically regulated by a phosphorylation reaction cascade brought about by protein kinase C (PKC), i.e. phorbol esters are able to decrease G_{Cl} up to 100% in the nanomolar range (Tricarico *et al*., 1991; De Luca *et al*., 1994). A PKC-mediated modulation of G_{Cl} has also been observed in mouse striated fibres (Brinkmeier & Jockusch, 1987). This is consistent with the idea of a common biochemical mechanism of muscle chloride channel regulation between species, likely involving a phosphorylation of the ClC-1 channel, as proved for other voltage-gated ion channels (Levitan, 1985). Nonetheless, the above described methodological limitations still make the clarification of a direct phosphorylation of the chloride channel unfeasible.

Similar intracellular modulatory pathways could also be involved in the reduction of muscle G_{Cl} observed in the ageing process. Phorbol esters applied to isolated muscle cause a more potent block of G_{Cl} in aged *vs* adult rats, suggesting that the low G_{Cl} of aged muscle could be due to an increased activity of phosphorylation pathways or to a higher channel sensitivity to them (De Luca *et al*., 1994). The G_{Cl} of aged rat muscle is also differently affected by specific channel ligands, such as the enantiomers of 2-(p-chlorophenoxy) propionic acid (CPP) (De Luca *et al*., 1992a,b). On adult rat muscle the S-(−) CPP blocks G_{Cl} dose-dependently with half maximal concentrations in the range of 10^{-5} M, whereas the R-(+) isomer shows a typical biphasic effect, increasing G_{Cl} at low concentrations (10^{-6} M) and decreasing it by 25%, at concentrations higher than 10^{-5} M (De Luca *et al*., 1992b). The G_{Cl} of aged rat muscle is less sensitive to the blocking action of the S(−)-CPP and lacks the typical biphasic response to R(+)-CPP, with only an increase of G_{Cl} being observed at all concentrations (De Luca *et al*., 1992b). We have recently demonstrated that a

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chronic treatment with growth hormone (GH) to aged rats counteracts the decrease of G_{Cl} and allows maintenance of the adult-like pharmacology to the enantiomers of CPP (De Luca *et al*., 1997). Once acutely applied to isolated muscle, insulin-like growth factor-1 (IGF-1), the GH peripheral mediator, completely restored the G_{Cl} of aged rats to the adult value (Florini *et al*., 1991; De Luca *et al*., 1997). This latter effect was inhibited by the prior incubation with okadaic acid, an inhibitor of the serine-threonine protein phosphatases (Cohen *et al*., 1990; De Luca *et al*., 1997). The above observations raise various questions. The main one is whether the stimulation of IGF-1 receptor in skeletal muscle contributes to the large G_{Cl} by counteracting the effect of phosphorylating pathways through the activation of a phosphatase. The other question is whether the modification of channel function due to phosphorylation can also account for the change in channel pharmacological properties. The present study aims at dealing with the above questions by reproducing on isolated skeletal muscle of adult rats a situation of enhanced PKC-induced phosphorylation of the chloride channels by the use of phorbol esters. The final aim of the present study is to shed light on the modulatory pathways that are essential for channel function in the native muscle fibres, and to evaluate the possibility of new therapeutic approaches for treating skeletal muscle disorders in which the decrease in G_{Cl} contributes to tissue malfunction.

Methods

Experimental procedure

Six to nine month-old male Wistar rats of 350–400 g were used for all the experiments. The electrophysiological experiments were made on isolated extensor digitorum longus (EDL) muscle. The muscles were removed under urethane anaesthesia (1.2 g kg^{-1}, i.p.) and soon after the removal of the muscle the rats, still anaesthetized, were killed by a further i.p. injection of an urethane overdose. The extensor digitorum longus muscle was stretched to about 1.5 times its resting length on a 3 mm plastic rod in a temperature controlled muscle chamber at 30°C and perfused with a physiological solution in the absence and in the presence of the test compounds.

Measurements of chloride conductance

The component chloride conductance (G_{Cl}) of extensor digitorum longus muscle fibres in the absence and in the presence of the test compounds was calculated from the cable parameters, and in particular from the membrane resistance (R_m) values, measured by standard cable analysis with the two intracellular microelectrode technique. In brief, a voltage-sensitive microelectrode (3 M KCl) was used to measure the membrane potential and the voltage deflection (electrotonic potential), monitored at two distances (0.5 mm and about 1 mm), in response to a hyperpolarizing square wave current pulse passed through a second electrode (2 M Kcitrate). In any experimental condition, the fibres sampled for recording of the cable parameters have membrane potential values ranging from −65 mV to −75 mV; in this condition no electronic setting of potential was necessary (Bryant & Conte Camerino, 1991). The bath electrode was a salt bridge made with 3 M KCl in agar. In agreement with previous observations (Bryant & Morales-Aguilera, 1971; Dulhunty, 1978), no significant junction potential occurred between electrodes and any of the bath solutions used. In fact no more than 2–3 mV difference in membrane potential values was observed between the recordings in normal and chloride-free physiological solution. Current pulse generation, acquisition of the voltage records and calculation of fibre constants (fibre diameter, membrane capacitance and membrane resistance) were carried out under computer control as detailed elsewhere (Bryant & Conte Camerino, 1991; De Luca *et al*., 1992a; 1994; 1997). In each fibre, the total membrane conductance, G_m, was $1/R_m$ in the normal physiological solution, whereas potassium conductance G_K was $1/R_m$ in the chloride-free physiological solution. The mean G_{Cl} was calculated as the mean G_m minus the mean G_K (De Luca *et al*., 1992a; 1994; 1997). The data is expressed as mean ± standard error of the mean (s.e.m.). The estimate of s.e.m. and N of G_{Cl} was calculated from the variance and N of G_m and G_K as described by Green & Margerison (1978). The estimates of s.e.m. and N of normalized values of G_{Cl} have been calculated as previously described (Green & Margerison, 1978; De Luca *et al*., 1992a,b). Significance between groups of means was evaluated by Student's *t*-test.

Solutions and drugs

The normal physiological solution had the following composition (mM): NaCl 148; KCl 4.5; $CaCl_2$ 2.0; $MgCl_2$ 1.0; NaH_2PO_4 0.44; $NaHCO_3$ 12 and glucose 5.55. The chloride free solution was made by equimolar substitution of methylsulphate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$ (De Luca *et al*., 1992a,b, 1994, 1997). The physiological solution was continuously bubbled with 95% O_2 and 5% CO_2 (pH = 7.2). The compounds tested were insulin-like growth factor-1 (IGF-1; Sigma Immunochemicals), porcine insulin, 4-β-phorbol-12,13-dibutyrate (4-β-PDB), 4-α-phorbol-12,13-didecanoate (4-α-PDD), okadaic acid, N-acetyl-sphingosine (C2-ceramide), sphingomyelin, dihydroceramide, and S(−) and R(+) enantiomers of 2-(p-chlorophenoxy) propionic acid (CPP). The S(−) and R(+) isomers of CPP were synthesized in our laboratories as previously described and prepared in stock solutions in normal or chloride-free physiological solutions (Bettoni *et al*., 1987; De Luca *et al*., 1992a,b, 1997). All the other chemicals used were obtained from Sigma (St. Louis, U.S.A.). IGF-1 was reconstituted in a stock solution of 10 μg in 0.1 M acetic acid, whereas insulin was dissolved in aqueous stock solutions. Stock solutions of all the other compounds tested were prepared in either dimethylsulphoxide (DMSO) (4-β-PDB, 4-α-PDB and okadaic acid) or in ethanol (C2-ceramide, sphingomyelin and dihydroceramide). The final concentrations that were tested were obtained by further dilution in normal and chloride-free solution, as needed. In agreement with previous results (Tricarico *et al*., 1991; De Luca *et al*., 1994) neither DMSO nor ethanol at much higher concentrations (0.5% and 1%, respectively) than those used during dilutions, exerted any effect on the parameters studied.

Drug exposure

In each preparation, after recordings of R_m in the normal physiological solution, a single concentration of the test compounds, either S(−)-CPP, R(+)-CPP, IGF-1, insulin, C2-ceramide, sphingomyelin or dihydroceramide was applied and incubated for an appropriate time before starting the recordings (De Luca *et al*., 1992a,b, 1997). After recording in the presence of the test compounds, a washout procedure enabled the establishment of the full reversibility of the drug effect. Afterwards 5 nM of 4-β-PDB or 0.25 μM of okadaic acid were applied and their effects were evaluated after 45 min of incubation (De Luca *et al*., 1994). Usually both 4-β-PDB and

okadaic acid produced effects that were irreversible upon washout and were constantly appreciable for more than 2 h. In these conditions the test compounds, at the same concentration tested in the normal physiological solution, were applied to evaluate their effect after the presumed channel phosphorylation. This procedure was also performed in the chloride-free physiological solution and no effect of any of the test drugs was found on G_K. Thus for the sake of brevity no description of drug effect on G_K will be reported in the Results section. To evaluate the involvement of PKC in the phorbol ester effect, the same procedure was performed in some preparations by using 4-α-PDD, the phorbol isomer inactive on the enzyme.

Results

Effect of IGF-1 on phorbol ester-mediated decrease of G_{Cl} of adult skeletal muscle

The application of 3.3 nM IGF-1 did not produce any change of macroscopic conductance to chloride ions (G_{Cl}) of EDL muscle fibres of adult rat in the normal physiological solution (Figure 1). The incubation of 5 nM of 4-β-PDB produced in twenty preparations tested a significant $35.8 \pm 2.8\%$ decrease of G_{Cl} within 45 min and such an effect was irreversible to washout for up to 2 h. As previously shown (Tricarico *et al*., 1991; De Luca *et al*., 1994), such a decrease in G_{Cl} was likely due to a modulation of channel function by a phosphorylation pathway via protein kinase C (PKC) activation. This was estimated in the present study by verifying that no effects on G_{Cl} were produced by the α isomer of the phorbol ester, known to be ineffective on PKC. In fact G_{Cl} was 2550 ± 257 μS cm^{-2} (n (of fibres) = 12) and 2744 ± 277 μS cm^{-2} ($n = 8$) in the absence and in the presence of 5 μM of 4-α-PDD, respectively. The application of IGF-1 caused within 10 min a complete restoration of G_{Cl} lowered by 4-β-PDB, to the value recorded in the absence of phorbol ester (Figure 1). The effect of IGF-1 was eliminated upon washout of the peptide from the bath, i.e. the effect of phorbol ester was again clearly detectable. Similarly to 4-β-PDB, the application of 0.25 μM okadaic acid, an inhibitor of serine-threonine protein phosphatases, produced a significant lowering of G_{Cl} of normal rat skeletal muscle (Figure 1), which was irreversible upon washout. However, the application of 3.3 nM IGF-1 on the muscle after the prior incubation of okadaic acid had no effect on G_{Cl} (Figure 1), suggesting that the availability of a phosphatase function is necessary for the somatomedin action. Insulin at equimolar concentrations of IGF-1 did not produce any effect on G_{Cl}, neither in the normal solution nor after the lowering of G_{Cl} with 5 nM 4-β-PDB; in this latter situation an increase of G_{Cl} was observed with high concentrations of insulin (10 μM; Table 1).

To test the hypothesis that IGF-1 counteracts the reduction in G_{Cl} induced by phorbol esters through a dephosphorylation reaction acting on the channel, we evaluated the effects of ceramide, known to exert some biological actions through the activation of an okadaic acid-sensitive protein phosphatase (Dobrowsky & Hannun, 1992). The application of 5 μM N-acetyl-sphingosine (C2-ceramide), a cell permeable analogue of natural ceramide, produced a slight and not significant increase of G_{Cl} in the normal physiological solution. However, when C2-ceramide was applied after prior incubation with 5 nM 4-β-PDB, a concentration-dependent increase of G_{Cl}

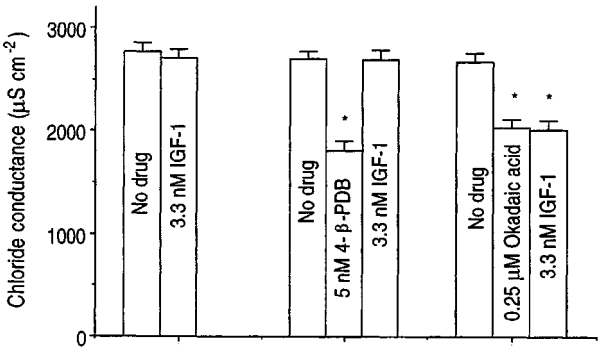

Figure 1 Effects of application of 3.3 nM insulin-like growth factor 1 (IGF-1) on membrane chloride conductance (G_{Cl}) of EDL muscle fibres of adult rats. From left to right, each group of bars shows the effects of IGF-1 on G_{Cl} in the normal physiological solution (no drug) and on G_{Cl} lowered by the prior incubation with either 5 nM 4-β-phorbol 12,13 dibutyrate (4-β-PDB) or 0.25 μM okadaic acid. Each bar is the mean ± s.e.mean of G_{Cl} from 12–63 fibres from 2–5 muscle preparations. *Significantly different with respect to the related control value (no drug) ($P < 0.05$ and less).

Table 1 Effects of insulin, dihydroceramide and sphingomyelin on G_{Cl} of rat EDL muscle fibres in the absence and presence of 4-β-PDB

		Normal solution		*4-β-PDB (5 nM)*	
Compound	*Concentration*	*N/n*	*G_{Cl} (μS cm^{-2})*	*N′/n′*	*G_{Cl} (μS cm^{-2})*
Insulin	0	4/41	2713 ± 116	3/24	1686 ± 89
	3.3 nM	2/16	2795 ± 150	2/22	1580 ± 117
	10 μM	1/8	2287 ± 127	1/7	2763 ± 84***
Dihydroceramide	0	2/14	3043 ± 166	2/20	1829 ± 157
	5 μM	2/16	2850 ± 160	2/11	1646 ± 184
Sphingomyelin	0	1/8	2735 ± 112	1/8	1242 ± 124
	3 μg ml^{-1} (10–30 min)			1/8	1698 ± 99*
	3 μg ml^{-1} (30–60 min)			1/5	1980 ± 186**

The compounds have been tested on resting chloride conductance (G_{Cl}), at the concentration shown in the table, in two different experimental conditions: in the absence (Normal Solution) and in the presence of 5 nM 4β-phorbol 12,13 dibutyrate (4-β-PDB). Each value is the means ± s.e.mean from N/n and N′/n′ (number of preparations/number of fibres) in the normal physiological solution and in the presence of 4-β-PDB, respectively. Each compound was incubated for 10 min and the recordings were taken within 30 min, with the exception of sphingomyelin, the effects of which were recorded within 30 min and 60 min of incubation. Due to a certain variability between preparations in the effect of 4-β-PDB, the effect of each compound has been compared to the value of G_{Cl} in the presence of phorbol ester recorded in the same set of experiments. Significantly different with respect to the related value in the absence of drug *$P < 0.02$; **$P < 0.01$; ***$P < 0.001$.

toward the control value was recorded (Figure 2). As observed with IGF-1, the effect of C2-ceramide was clearly appreciable after 10 min of incubation. Again, no effects were observed when the decrease of G_{Cl} was induced by a previous incubation of 0.25 μM okadaic acid (Figure 2). As shown in Table 1, dihydroceramide (5 μM), the ceramide inactive on phosphatase (Dobrowski & Hannun, 1992), was completely ineffective on G_{Cl} both in the normal physiological solution and in the presence of 4-β-PDB. The ceramide precursor sphingomyelin, at 3 μg ml^{-1}, caused a significant but slowly developing increase of the G_{Cl} lowered by phorbol ester (Table 1).

Effect of phorbol ester-mediated phosphorylation on the pharmacological modulation of G_{Cl} by the enantiomers of 2-(p-chlorophenoxy) propionic acid

The effects exerted by S(−)- and R(+)-CPP on muscle G_{Cl} in the normal physiological solution and in the presence of 5 nM 4-β-PDB are shown in Figures 3 and 4. As can be seen, in the normal physiological solution, the S(−)-CPP produced the classical graded concentration-related block of G_{Cl} with an EC_{50} of 30 μM. The phorbol ester-induced decrease of G_{Cl} was accompanied by a change in the effect produced by S(−)-CPP. In fact, at low concentrations we did not observe any decrease of G_{Cl}. Actually in the presence of 10 μM S(−)-CPP, G_{Cl} was significantly increased from 1707 ± 114 μS cm^{-2} ($n=14$) to 2418 ± 149 μS cm^{-2} ($n=19$) and a similar increase was observed also at 20 μM (Figure 3). A decrease of G_{Cl} by S(−)-CPP started to become appreciable at 50 μM, onward. The resultant concentration for producing a half-maximal block of G_{Cl} was clearly different from that observed in the normal physiological solution, being close to 80 μM.

Also the effects of R(+)-CPP were modified in the presence of phorbol esters. In fact, in the control condition the R(+)-CPP produced the typical biphasic effect, increasing significantly G_{Cl} at 3 μM and decreasing it by 20% at 100 μM. On the contrary, the R(+)-CPP produced only a marked increase of G_{Cl} once lowered by 4-β-PDB, at all the concentrations tested (Figure 4). At 0.3 μM the R(+)-CPP increased G_{Cl} up to the control value, and it produced a further 34% increase at 3 μM. No decrease of G_{Cl} by R(+)-CPP was observed at 100 μM; actually at this concentration of 61% increase of G_{Cl} was clearly detected. The effects of CPP enantiomers were rapidly reverted upon washout, both in the absence and in the presence of phorbol ester. The reversible effects of R(+)-CPP are shown in Figure 4.

Discussion

Phorbol ester-induced activation of PKC in the skeletal muscle of adult rat potently decreases in a concentration-dependent manner the resting macroscopic conductance to chloride ions (G_{Cl}) (Tricarico *et al*., 1991; De Luca *et al*., 1994). Several consensus sites for PKC phosphorylation are present in the carboxy-terminal region of the ClC-1 channel (Zhang *et al*.,

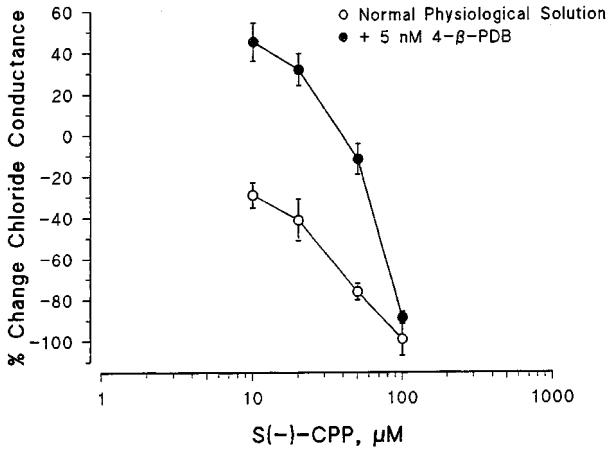

Figure 3 Effect of S-(−)-2-(p-chlorophenoxy) propionic acid (S(−)-CPP) on membrane chloride conductance (G_{Cl}) of EDL muscle fibres of adult rats in the normal physiological solution and after prior incubation with 4-β-phorbol-12,13 dibutyrate (4-β-PDB). In this latter condition the G_{Cl} value was lowered by about 30% as shown in Figures 1 and 2. In both experimental conditions the mean values of G_{Cl} recorded at each concentration of S-(−)CPP (from 10–29 fibres) have been normalized to the related G_{Cl} values in the absence of CPP (from 22–34 fibres). Thus, each point is the mean ± s.e.mean of the normalized % change of G_{Cl} (from 10–54 normalized values).

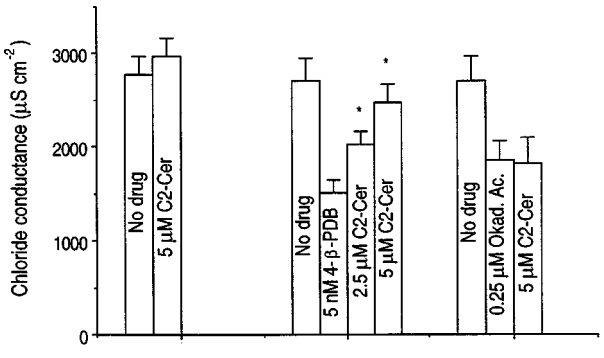

Figure 2 Effects of application of C2-Ceramide (C2-Cer; N-acetyl-sphingosine 2.5–5 μM) on membrane chloride conductance (G_{Cl}) of EDL muscle fibres of adult rats. From left to right, each group of bars show the effects of C2-Cer on G_{Cl} in normal physiological solution (no drug) and on G_{Cl} lowered by the prior incubation with either 5 nM 4-β-phorbol 12,13 dibutyrate (4-β-PDB) or 0.25 μM okadaic acid (Okad. Ac.). Each bar is the mean ± s.e.mean of G_{Cl} from 12–20 fibres from 2–3 muscle preparations. In the presence of either 4-β-PDB or okadaic acid the G_{Cl} was significantly decreased with respect to the related control value (No drug). *Significantly different with respect to the 4-β-PDB value ($P<0.05$ and less).

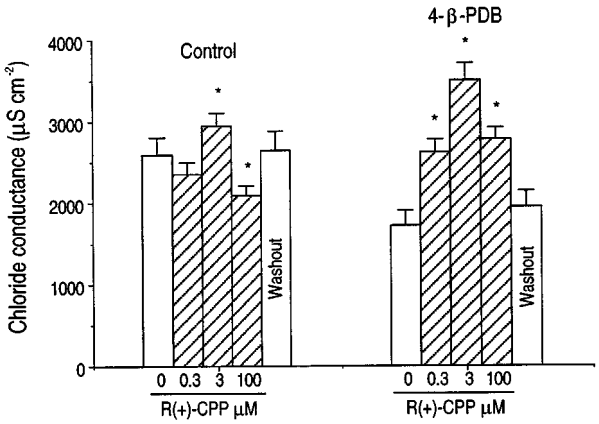

Figure 4 Effects of increasing concentrations of R(+)-2-(p-chlorophenoxy) propionic acid (R(+)-CPP) on membrane chloride conductance (G_{Cl}) of EDL muscle fibres in the normal physiological solution (control; left panel) and after prior incubation with 5 nM 4-β-phorbol 12,13 dibutyrate (4-β-PDB; right panel). Each bar is the mean ± s.e.mean value of G_{Cl} from 17–70 fibres.

1996), consistent with the notion that the phorbol ester-induced G_{Cl} reduction is due to a direct phosphorylation of the channel protein, as observed with other voltage-gated ion channels (Levitan, 1985). This is also supported by preliminary findings showing that the macroscopic chloride current of a heterologously expressed ClC-1 channel can be modulated by phorbol esters (Rosenbohlm *et al*., 1995), although these agents were less potent on recombinant chloride channels than on G_{Cl} of native muscle fibres. This latter observation also underlines the importance of the natural environment for a proper biochemical regulation of the chloride channel. Specific antibodies are not available to verify this hypothesis; thus we cannot rule out that the observed modulation of G_{Cl} by PKC is brought about by phosphorylation of an intermediate protein functionally or structurally connected with the channel (Isorm *et al*., 1994).

The PKC activation can be induced via a cholera-toxin sensitive G protein, suggesting that a physiological receptor controls the muscle chloride channel (De Luca *et al*., 1994). The present results show that the IGF-1 receptor controls an opposite dephosphorylating reaction that restores G_{Cl} in the case of enhanced activity of phosphorylating pathway. In fact, IGF-1 had no effect on G_{Cl} of a normal adult rat but it was able to increase this parameter lowered by a prior incubation with phorbol ester. Also, no effects of IGF-1 were observed on the G_{Cl} lowered by okadaic acid, an inhibitor of serine-threonine phosphatases. We have previously observed that IGF-1 counteracts the low G_{Cl} recorded in skeletal muscle of aged rats and that this effect is antagonized by okadaic acid (De Luca *et al*., 1997). All these observations corroborate the fact that in the two experimental conditions, IGF-1 acted on the channel through the same mechanism, i.e. the activation of an okadaic acid-sensitive protein phosphatase, such as PP1 or PP2A (Cohen *et al*., 1990). The results also imply that the activity of a phosphatase contributes to maintaining the chloride channel in a conductive state and that the decrease of G_{Cl} observed in aged animals is due to an excessive channel phosphorylation (De Luca *et al*., 1994). The apparent discrepancy concerning the reversibility of the IGF-1 effect observed in the present study can be explain by the irreversible PKC activation produced by the phorbol esters. This means that the equilibrium is rapidly shifted toward phosphorylation when IGF-1 is removed from the bath.

The specific involvement of the IGF-1 receptor was confirmed by the observation that insulin, at nanomolar concentration, failed to exert the effect observed with IGF-1. An IGF-1-like effect of insulin on G_{Cl} was observed with concentrations far exceeding those needed for a specific action through its own receptor, suggesting a possible cross-reaction with the homologous IGF-1 receptor (Florini *et al*., 1991).

An increasing amount of evidence indicates that IGF-1, as well as insulin, exerts short term effects in various tissues by modulating the activity of ion channels through phosphorylation-dephosphorylation pathways (Selinfreud & Blair, 1994; Shintani & Marunaka, 1996). In skeletal muscle, Delbono *et al*. (1997) have shown that IGF-1 plays a role in excitation-contraction coupling by acutely inducing a PKC-dependent phosphorylation of L-type calcium channels and consequently increasing channel activity. Such a mechanism appears to be impaired in striated fibres of aged rats (Renganathan *et al*., 1997). Insulin activates, through a PP1-dependent dephosphorylation, the Na^+/K^+ pump of skeletal muscle fibres (Begum *et al*., 1996); however the ability of IGF-1 to act *via* a serine-threonine phosphatase in skeletal muscle is a novel finding. This mechanism is corroborated by the observation that ceramide, known to activate an okadaic acid-sensitive serine-threonine phosphatase in various tissues among which skeletal muscle (Dobrowski & Hannun, 1992; Begum *et al*., 1996), produced the same effects of IGF-1 in our system.

The above observations also allow the speculation that ceramide plays a role in the transduction signal of the IGF-1 receptor. In fact, ceramide is the second messenger of various citokines and other endogenous factors and exerts a series of biological effects graded from differentiation to apoptosis (Hannun, 1994, 1996; Testi, 1996). IGF-1, in contrast with other growth factors, is known to stop proliferation in muscle culture and to induce muscle-specific differentiation (Florini *et al*., 1991; Coroneos *et al*., 1995). However, more experimental evidence is required to validate this hypothesis.

IGF-1 also exerts long-term effects on skeletal muscle (Florini *et al*., 1991) and likely on chloride channel. In fact, during ageing the decrease in muscle G_{Cl} is paralleled by a lower serum level of IGF-1 (De Luca *et al*., 1997). Furthermore, a chronic treatment with GH, peripherally acting through IGF-1, increases the low G_{Cl} typically observed in aged rat muscle and allows the maintenance of the adult-like response to the CPP enantiomers (De Luca *et al*., 1997). This latter effect has been explained with an *ex novo* synthesis of isoforms of chloride channels expressed in adult muscle; however, the present results suggest that the sensitivity to these specific channel ligands could also be due to a long-term control by IGF-1 on the phosphorylation state of the chloride channels. In fact we have found that the phosphorylation of the adult muscle chloride channel shifts its pharmacological profile toward that observed in aged animals. Thus, the experimentally phosphorylated chloride channels of adult rat muscle and the chloride channels of aged rat muscle have in common a decreased sensitivity to the blocking action of S(−)-CPP and a lack of the biphasic response by the R(+) isomer, that produced only an increase of G_{Cl} (De Luca *et al*., 1992b, 1997). Interestingly the R(+)-CPP can functionally antagonize the decrease of G_{Cl} due to phosphorylation, representing therefore a specific pharmacological agent.

Up to now we can only speculate about the mechanism by which phosphorylation can modify the channel sensitivity to the CPP enantiomers. According to our previous analysis, the opposite effects produced by the individual enantiomers in adult muscle fibres are due to the presence of two different receptors or binding sites able to enhance and decrease channel activity, respectively (De Luca *et al*., 1992b). It is possible that the channel modification brought about by phosphorylation involves a change in the conformation of the above sites so varying their affinity for the ligands. Another mechanism could be a phosphorylation-induced change in the voltage-dependent kinetic of the channel (Triggel, 1996).

In conclusion an hormonal-controlled balance between phosphorylation and dephosphorylation pathways modulates the function of muscle chloride channels. Taking into account that the large G_{Cl} is pivotal in ensuring the normal membrane excitability and muscle contractility (Lehmann-Horn & Rüdel, 1996), the activity of an endogenous phosphatase may be important in maintaining the channel in a conductive state as a protective mechanism in physiological situations that can lead to a decrease of G_{Cl}, i.e. sudden exercise and/or muscle fatigue (Haller & Bertocci, 1994). The possibility of pharmacologically interfering on these dynamic pathways could open new approaches to improving muscle function.

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References

BEGUM, N., RAGOLIA, L. & SRINIVASAN, M. (1996). Effect of tumor necrosis factor-alpha on insulin-stimulated mitogen-activated protein kinase cascade in cultured rat skeletal muscle cells. *Eur. J. Biochem.,* **238,** 214 – 220.

BETTONI, G., LOIODICE, F., TORTORELLA, V., CONTE CAMERINO, D., MAMBRINI, M., FERRANNINI, E. & BRYANT, S.H. (1987). Stereospecificity of the chloride ion channel: the action of chiral clofibric acid analogues. *J. Med. Chem.,* **30,** 1267 – 1270.

BRINKMEIER, H. & JOCKUSCH, H. (1987). Activators of protein kinase C induce myotonia by lowering chloride conductance in muscle. *Biochem. Biophys. Res. Commun.,* **148,** 1383 – 1389.

BRYANT, S.H. & CONTE CAMERINO, D. (1991). Chloride channel regulation in the skeletal muscle of normal and myotonic goats. *Pflügers Arch,* **417,** 605 – 610.

BRYANT, S.H. & MORALES-AGUILERA, A. (1971). Chloride conductance in normal and myotonic muscle fibres and the action of monocarboxylic aromatic acids. *J. Physiol. (Lond),* **219,** 367 – 383.

CHUA, M. & BETZ, W.I. (1991). Characterization of ion channels on the surface membrane of adult rat skeletal muscle. *Biophys. J.,* **59,** 1251 – 1260.

COHEN, P., HOLMES, C.F.B. & TSUKITANI, Y. (1990). Okadaic acid: a new probe for the study of cellular regulation. *Trends Biol. Sci.,* **15,** 98 – 102.

CORONEOS, E., MARTINEZ, M., MCKENNA, S. & KESTER, M. (1995). Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. *J. Biol. Chem.,* **270,** 23305 – 23309.

DELBONO, O., RENGANATHAN, M. & MESSI, M.L. (1997). Regulation of mouse skeletal muscle L-type Ca^{2+} channel by activation of the insulin-like growth factor-1 receptor. *J. Neurosci.,* **17,** 6918 – 6928.

DE LUCA, A., PIERNO, S., COCCHI, D. & CONTE CAMERINO, D. (1997). Effects of chronic growth hormone treatment in aged rats on the biophysical and pharmacological properties of skeletal muscle chloride channels. *Br. J. Pharmacol.,* **121,** 369 – 374.

DE LUCA, A., TORTORELLA, V. & CONTE CAMERINO, D. (1992a). Chloride channels of skeletal muscle from developing, adult and aged rats are differently affected by enantiomers of 2-(p-chlorophenoxy) propionic acid. *Naunyn-Schmied. Arch Pharmacol.,* **346,** 601 – 606.

DE LUCA, A., TRICARICO, D., PIERNO, S. & CONTE CAMERINO, D. (1994). Aging and chloride channel regulation in rat fast-twitch muscle fibres. *Pflügers Arch,* **427,** 80 – 85.

DE LUCA, A., TRICARICO, D., WAGNER, R., BRYANT, S.H., TORTORELLA, V. & CONTE CAMERINO, D. (1992b). Opposite effects of enantiomers of clofibric acid derivative on rat skeletal muscle chloride conductance: antagonism studies and theoretical modeling of two different receptor sites interactions. *J. Pharmacol. Exp. Ther.,* **260,** 364 – 369.

DOBROWSKY, R.T. & HANNUN, Y.A. (1992). Ceramide stimulates a cytosolic protein phosphatase. *J. Biol. Chem.,* **267,** 5048 – 5051.

DULHUNTY, A.F. (1978). The dependence of membrane potential on extracellular chloride concentration in mammalian skeletal muscle fibres. *J. Physiol. (Lond),* **276,** 67 – 82.

FAHLKE, C., BECK, C.L. & GEORGE, Jr, A.L. (1997). A mutation in autosomal dominant myotonia congenita affects pore properties of the muscle chloride channel. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 2729 – 2734.

FLORINI, J.R., EWTON, D.Z. & MAGRI, K.A. (1991). Hormones, growth factors and myogenic differentiation. *Ann. Rev. Physiol.,* **53,** 201 – 216.

GREEN, J.R. & MARGERISON, D. (1978). *Statistical Treatment of Experimental Data*, pp. 86 – 88, New York, Elsevier.

HALLER, R.G. & BERTOCCI, L.A. (1994). Exercise evaluation of metabolic myopathies. In *Myology* ed. Engel, A.G. & Franzini-Armstrong, C. chapt. 33, pp. 807 – 821. New York: McGraw Hill, Inc.

HANNUN, Y.A. (1994). The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.,* **269,** 3125 – 3128.

HANNUN, Y.A. (1996). Functions of ceramide in coordinating cellular responses to stress. *Science,* **274,** 1855 – 1859.

ISORM, L.L., DE JONGH, K.S. & CATTERALL, W.A. (1994). Auxiliary subunits of voltage-gated ion channels. *Neuron,* **12,** 1183 – 1194.

LEHMANN-HORN, F. & RÜDEL, R. (1996). Molecular pathophysiology of voltage gated ion channels. *Rev. Physiol. Biochem. Pharmacol.,* **128,** 195 – 268.

LEVITAN, I.B. (1985). Phosphorylation of ion channels. *J. Membr. Biol.,* **87,** 177 – 190

PUSCH, M. & JENTSCH, T.J. (1994). Molecular physiology of voltage-gated chloride channels. *Physiol. Rev.,* **74,** 813 – 827.

PUSCH, M., STEINMEYER, K. & JENTSCH, T.J. (1994). Low single channel conductance of the major skeletal muscle chloride channel, ClC-1. *Biophys. J.,* **66,** 149 – 152.

RENGANATHAN, M., SONNTAG, W.E. & DELBONO, O. (1997). L-type Ca^{2+} channel-insulin-like growth factor-1 receptor signaling impairment in aging rat skeletal muscle. *Biochem. Biophys. Res. Comm.,* **235,** 784 – 789.

ROSENBOHLM, A., GEORGE, A.L., RÜDEL, R. & FAHLKE, C. (1995). Effect of activation of protein kinase C (PKC) on the muscle chloride channel, ClC-1, studied in native and heterologous expression system. *Pflügers Arch,* **429,** R25.

SELINFREUD, R.H. & BLAIR, L.A.C. (1994). Insulin-like growth factor-I induces a rapid increase in calcium currents and spontaneous membrane activity in clonal pituitary cells. *Mol. Pharmacol.,* **45,** 1215 – 1220.

SHINTANI, Y. & MARUNAKA, Y. (1996). Regulation of single Cl^- channel conductance by insulin and tyrosine phosphatase. *Biochem. Biophys. Res. Commun.,* **218,** 142 – 147.

TESTI, R. (1996). Sphingomyelin breakdown and cell fate. *Trends Biol. Sci.,* **21,** 468 – 471.

TRICARICO, D., CONTE CAMERINO, D., GOVONI, S. & BRYANT, S.H. (1991). Modulation of rat skeletal muscle chloride channels by activators and inhibitors of protein kinase C. *Pflügers Arch,* **418,** 500 – 503.

TRIGGLE, D.J. (1996). Ion channels as pharmacological receptors: the chirality of drug interactions. *Chirality,* **8,** 35 – 38.

ZHANG, J., GEORGE, Jr, A.L., GRIGGS, R.C., FOAUND, G.T., ROBERTS, J., KWIECINSKI, H., CONNOLLY, A.M., PTÁCEK, L.J. (1996). Mutations in the human skeletal muscle chloride channel gene (CLCN1) associated with dominant and recessive myotonia congenita. *Neurology,* **47,** 993 – 998.

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