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European Journal of Pharmacology 362 (1998) 221–227

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Uridine triphosphate-sensitive pathway of Ca^{2+} release from the sarcoplasmic reticulum of rat skeletal muscle fibers

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Received 7 May 1998; revised 9 October 1998; accepted 16 October 1998

Abstract

The pyrimidine nucleotide, uridine triphosphate (UTP), was tested with skinned skeletal muscle fibers in order to investigate the UTP-sensitive pathway of Ca^{2+} release from the sarcoplasmic reticulum. The presence of ryanodine (200 μM), ruthenium red (10 μM) or heparin (2.5 mg/ml) did not affect the tension elicited in the presence of UTP, demonstrating that the UTP-induced Ca^{2+} release involved neither ryanodine nor inositol triphosphate-sensitive channels. Drugs such as compound 48/80 or cyclopiazonic acid used to inhibit Ca^{2+}-ATPase in its reverse function appeared to be, respectively, non-specific or without any inhibitory effect on the tension induced by UTP. Finally, the UTP-induced tension as well as the trifluoperazine-induced tension were abolished in the presence of spermidine (50 mM), supporting the hypothesis that the UTP-sensitive pathway of the SR Ca^{2+} release might occur through the uncoupled calcium ATPase.

Keywords: Ca^{2+} release; Skinned muscle fiber; Sarcoplasmic reticulum; UTP-induced tension

1. Introduction

The sarcoplasmic reticulum regulates the cytoplasmic Ca^{2+} concentration of skeletal muscle cells and thereby controls muscular contraction and relaxation. Indeed, the sarcoplasmic reticulum is able to sequester Ca^{2+} via Ca^{2+}-ATPases and release the stored Ca^{2+} ions through channels like the ryanodine receptors. These two sarcoplasmic reticulum functions are regulated by ATP since Ca^{2+} uptake requires ATP hydrolysis and ryanodine receptors are activated by this nucleotide. Other nucleotides display variable but lower effectiveness as substitutes for ATP. For instance, the sarcoplasmic reticulum Ca^{2+} uptake and the affinity of Ca^{2+}-ATPase for the nucleotide decreased when GTP or ITP replaced ATP (De Meis and de Mello, 1973). Moreover, Kakuta (1984) reported that ITP, CTP and UTP also evoked Ca^{2+} release from the sarcoplasmic reticulum with an efficiency which decreased in the order ITP, CTP, UTP. Hasselbach (1978) also reported that, in presence of ADP, P_i and a Ca^{2+}-gradient, Ca^{2+}-ATPase could operate on reversal and catalyzed the synthesis of ATP from ADP and P_i. Ca^{2+}-ATPase can also be uncoupled by different drugs such as phenothiazines and assimilated to a channel. Indeed, De Meis (1991) demonstrated that trifluoperazine increased the efflux of Ca^{2+} through the uncoupled ATPase and that this activation was antagonized by spermidine.

Previous experiments with skinned rat muscle fibers have shown that UTP, when replacing ATP, reduces sarcoplasmic reticulum Ca^{2+} uptake and induces Ca^{2+} release out of the Ca^{2+}-loaded sarcoplasmic reticulum (Suarez-Kurtz et al., 1993, 1995). However, the UTP-induced tension persists after treatment with ruthenium red, suggesting that the UTP-sensitive pathway might be different from the sarcoplasmic reticulum terminal cisternae Ca^{2+} channels identified as ryanodine receptors. The functional properties of the latter have been well described; for instance, activation by micromolar Ca^{2+} concentrations, ATP, caffeine and inhibition by 100 μM ryanodine and ruthenium red. In the present experiments, we decided to study the UTP-sensitive pathway of Ca^{2+} release using pharmacological approaches. We showed that UTP acts neither on the ryanodine receptor nor on the inositol triphosphate (IP_3) receptor and that the UTP-sensitive Ca^{2+} release occurs through uncoupling of Ca^{2+}-ATPase.

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00756-0

2. Materials and methods

2.1. Materials

Small bundles of fibers were dissected from the extensor digitorum longus of the rat, chemically skinned by exposure to an EGTA-containing 'skinning' solution, before storage at −20°C in the presence of glycerol until use (Wood et al., 1975). As previously described (Wood et al., 1975; Eastwood et al., 1979) this procedure allows the sarcolemma to be made permeable to external solutions without affecting either the functions of the sarcoplasmic reticulum or those of the contractile proteins. On the day of an experiment, a segment of a single fiber was isolated from the skinned bundles and was mounted in an experimental chamber containing the skinning solution. The temperature of the room was maintained at 20 ± 1°C. To record isometric tension, one end of the fiber was attached to a clamp connected to a force-displacement transducer and the other end was fixed to a hook connected to a micromanipulator. The relaxed fiber was stretched to a sarcomere length of 2.60 μm, measured by the light diffraction of a He–Ne laser beam directed perpendicularly to the long axis of the fiber. This sarcomere length was maintained and was checked throughout the entire experiment. The transducer signals were amplified and recorded on Gould (Cleveland, OH) polygraphs.

2.2. Solutions

The composition of all the solutions was calculated using the computer program developed by Fabiato (1988), using the constants given by Fabiato and Fabiato (1979). Association constants for CaEGTA and MgATP were those reported by Orentlicher et al. (1977). When UTP replaced ATP, association constants reported by Martell and Smith (1974) were used. MOPS (3-(*N*-morpholino) propanesulfonic acid) was used as pH buffer (pH 7.0). The control bathing medium ('washing' solution designated W) had the following composition (in mM): 185 K-propionate, 2.5 K_2Na_2ATP, 2.5 Mg acetate, 10 MOPS. Subsequently, 50 μM K_2EGTA was added to this solution to reduce the concentration of contaminating Ca^{2+}. The skinning solution and the 'relaxing' solution (designated R) had the same composition (in mM): 170 K-propionate, 2.5 K_2Na_2ATP, 2.5 Mg acetate, 5 K_2EGTA, 10 MOPS. Solutions used to activate the myofibrils directly ('activating' solution of pCa 4.2) or to load Ca^{2+} into the SR ('loading' solution of pCa 6.5) were prepared by partially replacing K_2EGTA in the relaxing solution (R) by CaK_2EGTA to obtain different ratios of K_2EGTA/CaK_2EGTA, while keeping the total [EGTA] constant at 5 mM. Solution W in which ATP (2.5 mM) was replaced by UTP is designated W-UTP. This solution, W-UTP, was always applied twice in order to eliminate ATP contamina-

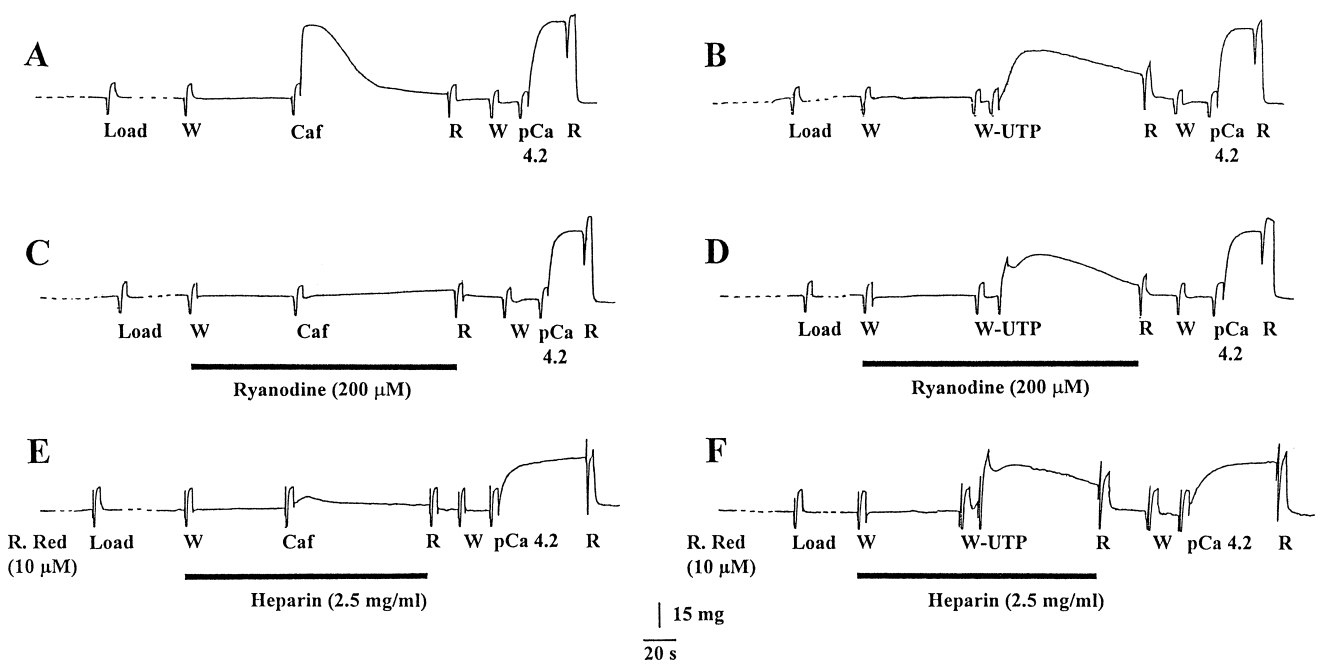

Fig. 1. Effects of ryanodine and heparin on the caffeine- and UTP-induced tensions, in skinned fibers of rat extensor digitorum longus muscles. The experimental protocol for each panel consisted of initially loading Ca^{2+} into the SR (Load) during a 3-min exposure to a pCa 6.5 solution. After being rinsed in solution W, the fiber was challenged with 20 mM caffeine in solution W (Caf, in A) or 2.5 mM UTP in solution W (W-UTP, in B). The fiber was relaxed in solution R and washed in solution W, then exposed to an ATP-containing solution of pCa 4.2 to record T_{max} before being relaxed in solution R. In C and D (same fiber as in A and B), 200 μM ryanodine was added to the washing and tension-elicited solutions, as indicated by black lines. Before experiments E and F, the fiber was exposed to ruthenium red (R. Red, at 10 μM in solution R) for 10 min. A total of 2.5 mg/ml of heparin was then added after the SR load, as shown by black lines.

tion from the preceding solution. The UTP-induced tension is inhibited in a dose-dependent manner by ATP (Suarez-Kurtz et al., 1995). Moreover, if an ATP regenerating system made of 10 mM creatine phosphate and 15 U/ml creatine phosphokinase was added to the different solutions, the results were not modified. All the solutions contained enough nucleotide and were applied once before being discarded. The experimental conditions were thus identical in the presence of ATP or UTP. Caffeine, compound 48/80, cyclopiazonic acid, heparin, ruthenium red, ryanodine, spermidine and trifluoperazine were then added to the experimental solutions, as indicated. All chemicals were from Sigma (Sigma, St Louis, MO, USA), except UTP which was provided by Wyeth Laboratories (Paris, France).

2.3. Experimental protocols

At the beginning and at the end of each experimental protocol, T_{max}, the maximal contractile response of the fibers when exposed to an ATP-containing solution of pCa 4.2, was recorded. The functional properties of the sarcoplasmic reticulum were evaluated from the development of the caffeine-induced tension. Ca^{2+} release was then tested in presence of UTP. The procedure was carried out in 5 steps: (1) The fiber was initially exposed to 20 mM caffeine in solution R to deplete sarcoplasmic reticulum Ca^{2+} stores. (2) After two washes with W, the fiber was soaked in the loading solution of pCa 6.5 for 3 min, conditions which allowed maximal loading of the sarcoplasmic reticulum (Suarez-Kurtz et al., 1995). (3) The fiber was rinsed again in W, for 10, 30 or 60 s. (4) Release of the stored Ca^{2+} was induced by a releasing solution, using different agents such as caffeine (20 mM), trifluoperazine (100 μM) or UTP (2.5 mM) in solution W. (5) Finally, the fiber was relaxed using solution R. Drugs (compound 48/80, cyclopiazonic acid, heparin, ryanodine or spermidine) were added after the normal load during step 3 for various durations depending on drug properties and during step 4.

The amplitude of tensions was expressed relative to T_{max} and was taken to be more representative of the release mechanism than the measurement of the area under the tension curve. Data from pooled experiments are presented as means ± S.E. Student's *t*-test was used for statistical analysis of the data.

3. Results

3.1. Ca^{2+} release through ryanodine receptor

In skinned extensor digitorum longus fibers, after sarcoplasmic reticulum loading, caffeine (20 mM) induced marked tension (Fig. 1A) which reached 91.6 ± 1.8% of T_{max} ($n = 6$). Under similar loading conditions, UTP

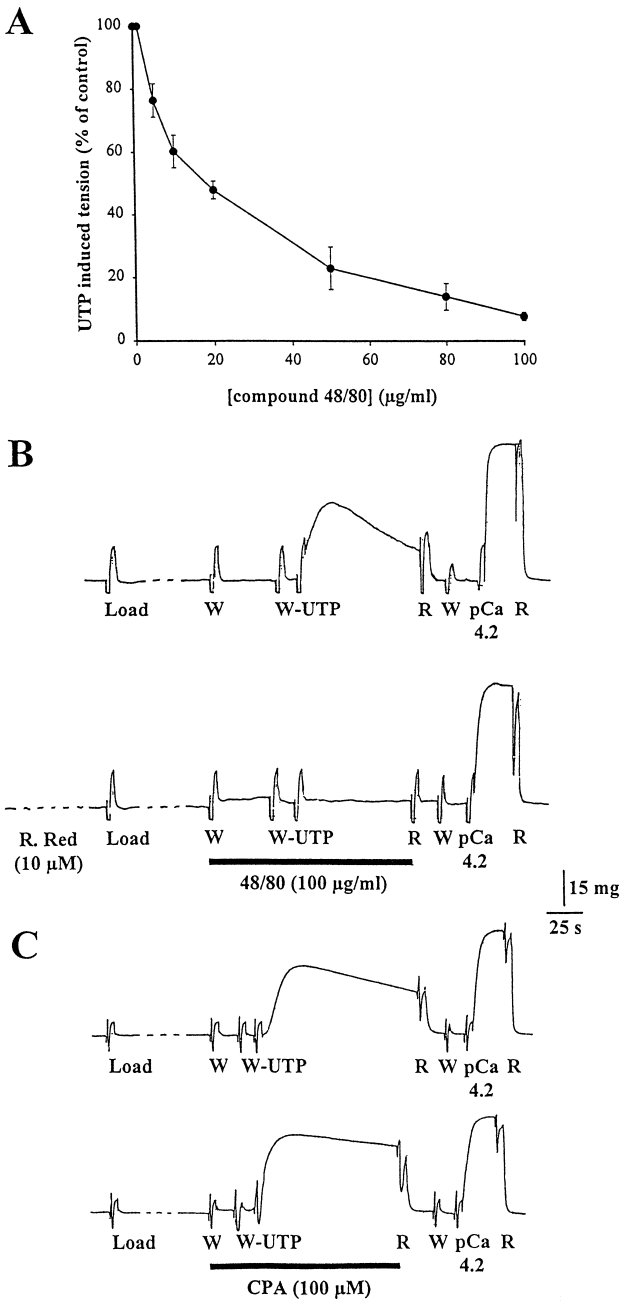

Fig. 2. Effect of compound 48/80 and cyclopiazonic acid (CPA) on the UTP-induced tension. The fiber was soaked in a pCa 6.5 solution for 3 min, then washed in solution W before challenge with 2.5 mM UTP in solution W, and relaxation with a solution R. T_{max} was recorded as previously described in Fig. 1. After recording of control tension, the fiber was treated with ruthenium red (10 μM in solution R, for 10 min). (A) Dose–response curve for the effect of compound 48/80 on the UTP-induced tension. Values are means ± S.E. of two experiments. Compound 48/80 was added after the sarcoplasmic reticulum load at various concentrations. (B) Illustration of the maximal inhibition of compound 48/80 at 100 μg/ml. The presence of drug is indicated by a black line. (C) Effect of cyclopiazonic acid (CPA) on the UTP-induced tension. 100 μM CPA was added to solutions W and W-UTP as indicated by the black line.

(2.5 mM) also induced a tension (Fig. 1B) with a mean amplitude equal to $62.9 \pm 6.2\%$ of T_{max} ($n = 6$). When these experiments were performed in the presence of ryanodine (200 μM), the caffeine-induced tension was abolished (Fig. 1C) while the amplitude of the tension induced by UTP remained similar (Fig. 1D). In the same way, after the exposure to 10 μM ruthenium red (not illustrated), the caffeine-elicited tension represented $2.5 \pm 1.2\%$ of T_{max} ($n = 6$) while the amplitude of the UTP-induced tension was not affected and remained at $56.5 \pm 3.7\%$ of T_{max} ($n = 6$) ($P > 0.05$), not significantly different from the control value in absence of the drug, (Fig. 1B). The blockade of the caffeine-induced tension through the ryanodine channel was irreversible as already described (Salviati and Volpe, 1988).

3.2. Ca^{2+} release through IP_3 receptor

IP_3-sensitive calcium channels can be selectively inhibited by heparin which is also well known to stimulate ryanodine receptors (Bezprozvanny et al., 1993). We checked for a possible effect of UTP on the IP_3 pathway using heparin at a concentration of 2.5 mg/ml, previously demonstrated to be sufficient to inhibit the IP_3 calcium release pathway in skeletal muscle (Valdivia et al., 1992; Lopez and Terzic, 1996). Moreover, heparin was applied in the presence of ruthenium red in order to avoid any activation of the caffeine-controlled ryanodine receptors (Fig. 1E). Under these conditions, the UTP-induced tension persisted (Fig. 1F) while tension induced by IP_3 ($85.9 \pm 5.9\%$ of T_{max}, $n = 2$ at 200 μM) was reduced by $67.8 \pm 5.4\%$ at this concentration of heparin ($n = 2$, data not shown). No inhibition of UTP-induced tension was obtained using 1 and 5 mg/ml heparin. We did not use higher concentrations of this drug since it affected the contractile proteins by reducing their affinity to Ca^{2+} (data not shown).

3.3. Ca^{2+} release through Ca^{2+}-ATPase

We investigated whether UTP could induce Ca^{2+} release through Ca^{2+}-ATPase since it has been shown that

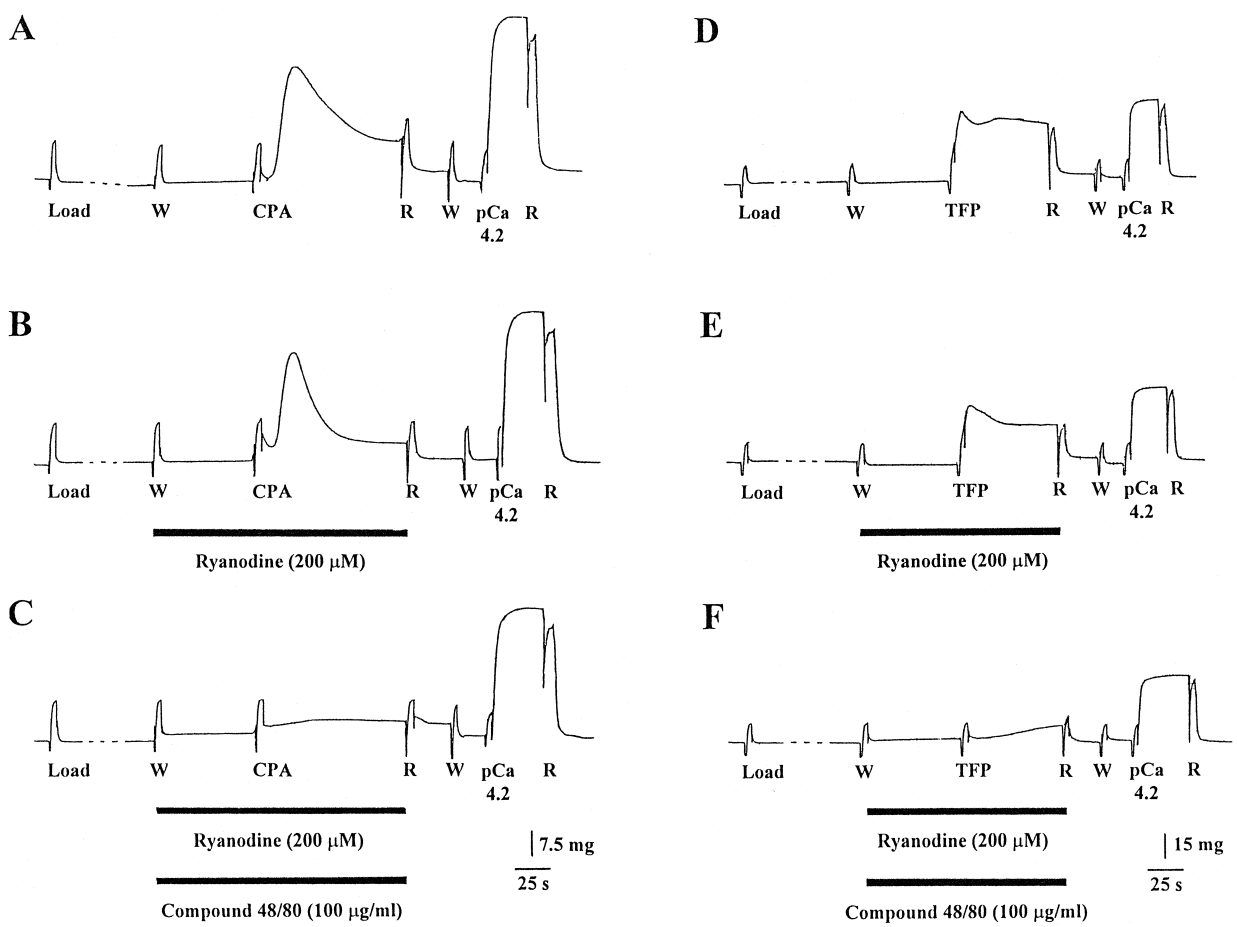

Fig. 3. Effects of ryanodine and compound 48/80 on the tension elicited by cyclopiazonic acid (CPA) and trifluoperazine (TFP). After the sarcoplasmic reticulum maximal load (pCa 6.5; 3 min), the fiber was washed for 1 min in solution W. The fiber was then challenged with 100 μM CPA or 100 μM TFP in solution W. The fiber was relaxed in solution R and T_{max} was recorded as previously described for Fig. 1. (A) Control tension induced by 100 μM CPA. (B) CPA-induced tension in the presence of 200 μM ryanodine. (C) CPA-induced tension in the presence of 200 μM ryanodine and 100 μg/ml compound 48/80. (D) control tension induced by 100 μM TFP. (E) TFP-induced tension in the presence of 200 μM ryanodine. (F) TFP-induced tension in the presence of both 200 μM ryanodine and 100 μg/ml compound 48/80. Black lines indicate the presence of drugs.

Ca^{2+}-ATPase can mediate Ca^{2+} efflux from the sarcoplasmic reticulum in either a coupled (reverse of the pump) with respect to catalytic events, or an uncoupled, mode (De Meis, 1991).

3.3.1. Reverse of the pump

Compound 48/80 has been described as an inhibitor of the reverse function of Ca^{2+}-ATPase and as a stimulator of the Ca^{2+} release channels (Vale, 1990). Therefore, the effect of UTP was tested in the presence of both ruthenium red and 48/80 as illustrated in Fig. 2. The control tension with UTP was first recorded. Then exposure to ruthenium red (10 μM) preceded the sarcoplasmic reticulum maximal load. When the load was achieved, the UTP-induced release was elicited in the presence of compound 48/80. Various concentrations of this drug were tested (from 0.1 to 100 μg/ml). The concentration-effect curve (Fig. 2A) demonstrated that compound 48/80 reduced the UTP-induced tension with complete inhibition at 100 μg/ml (Fig. 2B). Mean amplitudes of the UTP-induced tension (expressed in % of T_{max}) were 48.0 ± 2.0 ($n = 4$) and 4.0 ± 1.8 ($n = 4$) in the absence or presence of 48/80 (100 μg/ml), respectively ($P < 0.05$).

Cyclopiazonic acid, a well-known inhibitor of the Ca^{2+}-ATPase pump (Goeger et al., 1988) but also described as an inhibitor of the reverse of Ca^{2+}-ATPase (Du et al., 1996), was tested on the UTP-induced tension as shown in Fig. 2C. Under our experimental conditions, the presence of cyclopiazonic acid did not reduce the UTP-induced tension. On the contrary, the presence of cyclopiazonic acid amplified this tension; thus the mean amplitude of the UTP-induced tension increased from 56.0 ± 7.9 to 83.1 ± 1.3% of T_{max} ($n = 3$, $P < 0.05$) and the tension clearly appeared to increase more rapidly. Moreover, after the sarcoplasmic reticulum load, the wash containing cyclopiazonic acid had to be brief (about 10 s) otherwise a tension was recorded. Indeed, cyclopiazonic acid itself was able to induce tension (Fig. 3A) with an amplitude 77.5 ± 10.2% of T_{max} ($n = 3$) and which was not suppressed by 200 μM ryanodine (Fig. 3B). On the contrary, the cyclopiazonic acid-induced tension was reduced by compound 48/80 (100 μg/ml). The amplitude decreased significantly to 9.7 ± 4.9% ($P < 0.05$), corresponding to 88.9 ± 5.6% inhibition ($n = 3$) (Fig. 3C).

3.3.2. Uncoupling of the pump

The phenothiazine, trifluoperazine (100 μM), has been described as an activator of Ca^{2+} efflux through the uncoupled Ca^{2+}-ATPase (De Meis, 1991). After sarcoplasmic reticulum loading, trifluoperazine (100 μM) induced a marked tension (Fig. 3D) which reached 84.1 ± 1.7% of T_{max} ($n = 3$). In the presence of ryanodine (200 μM) (Fig. 3E), the tension elicited by trifluoperazine was unchanged and the mean amplitude was 87.9 ± 7.0% of

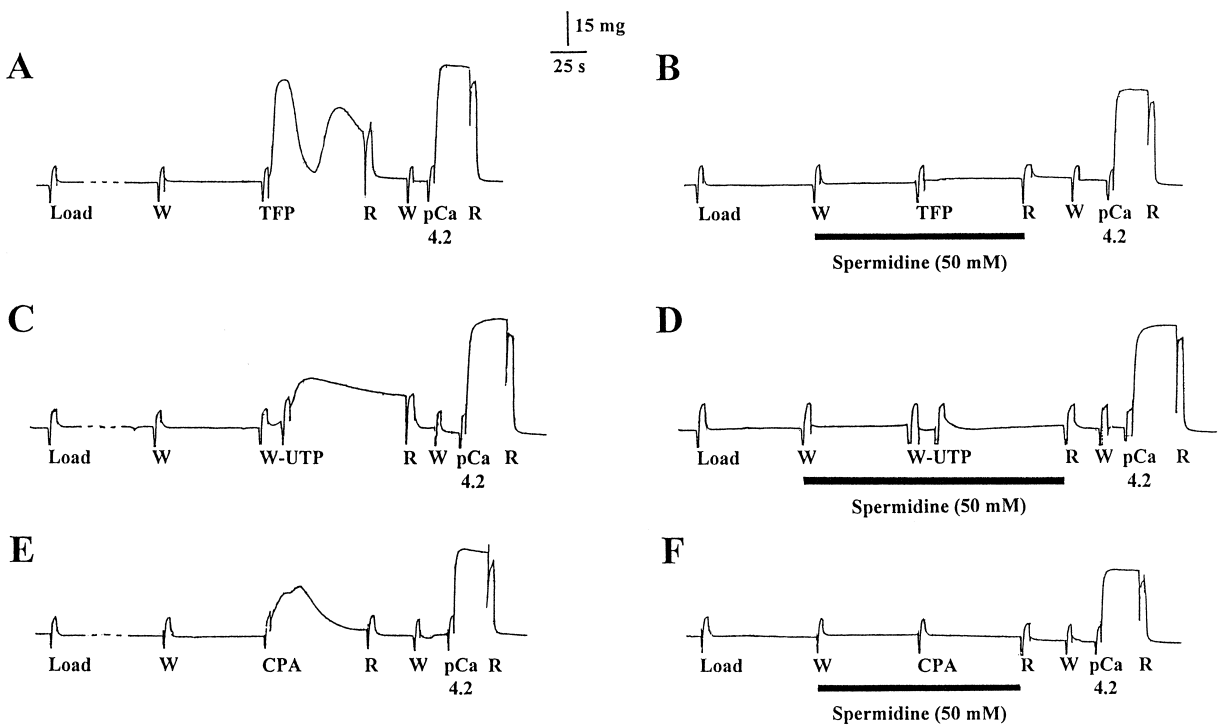

Fig. 4. Effect of spermidine on the trifluoperazine (TFP)-, UTP- and cyclopiazonic acid (CPA)-induced tensions. After the sarcoplasmic reticulum maximal load (pCa 6.5; 3 min), the fibers were washed for 1 min in solution W. One series was then exposed to 100 μM trifluoperazine in solution W (TFP, A and B) whereas the others were challenged with 2.5 mM UTP in solution W (W-UTP, C and D) or with 100 μM cyclopiazonic acid in solution W (CPA, E and F). The fibers were relaxed in solution R, and T_{max} was recorded as previously described for Fig. 1. After recording of control tensions (in A, C and E), the same protocol was repeated in the presence of 50 mM spermidine as indicated by black lines (in B, D and F).

T_{max} ($n = 3$). On the contrary, application of compound 48/80 (100 μg/ml) significantly reduced, by 64.6 ± 6.7% the trifluoperazine-induced tension, decreasing the amplitude to 31.7 ± 7.9% of T_{max} ($n = 3$) ($P < 0.05$) (Fig. 3F).

Spermidine has been used to inhibit the efflux of Ca^{2+} caused by phenothiazines like trifluoperazine (De Meis, 1991). The effect of spermidine was tested on the trifluoperazine- and UTP-induced tensions as illustrated in Fig. 4. Control tensions were first recorded. Trifluoperazine (100 μM) induced a marked tension (Fig. 4A) representing 84.5 ± 3.3% of T_{max} ($n = 5$). The mean amplitude of the UTP-induced tension (Fig. 4C) was equal to 40.5 ± 5.1% of T_{max} ($n = 8$). In the presence of 50 mM spermidine, the trifluoperazine- and UTP-induced tensions were both greatly reduced (Fig. 4B and D) since mean amplitudes (expressed in % of T_{max}) were 10.7 ± 7.3 ($n = 5$, $P < 0.05$) and 2.1 ± 0.8 ($n = 8$, $P < 0.05$), corresponding respectively to 87.3 ± 7.3 and 94.6 ± 2.1% of the inhibition by spermidine. The effect of this drug was also tested on the cyclopiazonic acid-induced tension (Fig. 4E and F). In the absence of spermidine (50 mM), the tension elicited by cyclopiazonic acid (100 μM) had a mean amplitude of 64.7 ± 4.2% of T_{max} ($n = 5$) and was abolished in the presence of the drug. The mean amplitude was 2.0 ± 1.3% of T_{max} ($P < 0.05$) that corresponded to 96.9 ± 2.0% of the inhibition by spermidine.

4. Discussion

UTP has already been described as a nucleotide able to induce a tension dependent on an increased release and decreased uptake of Ca^{2+} by the sarcoplasmic reticulum as well as on increased sensitivity of contractile proteins to Ca^{2+} (Suarez-Kurtz et al., 1995). In the present study, the different pathways of calcium release from the sarcoplasmic reticulum were considered in order to identify the UTP-sensitive pathway.

First, ryanodine and ruthenium red were used since these two inhibitors have been recognized to specifically act on ryanodine receptors (Coronado et al., 1994). With 200 μM ryanodine or 10 μM ruthenium red, the caffeine-elicited tension disappeared, while the same treatment had no effect on the UTP-induced tension suggesting that the sarcoplasmic reticulum Ca^{2+} release due to UTP did not involve ryanodine receptors. These results are in good agreement with those of previous experiments which reported no stimulating effect of UTP on Ca^{2+} release channels incorporated into lipid bilayers (Suarez-Kurtz, 1994). Moreover, based on Volpe et al. (1986) and Alves and De Meis (1986), it should be emphasised that ruthenium red had no effect on the sarcoplasmic reticulum Ca^{2+}-load under our experimental conditions since the amplitude of the UTP-induced tension was not significantly different from the control tension.

Another calcium release pathway involving IP_3 receptors was considered. Previous experiments with heavy sarcoplasmic reticulum vesicles of rabbit skeletal muscles (Valdivia et al., 1992) or skinned skeletal fibers (Lopez and Terzic, 1996) have shown that the Ca^{2+} release induced by IP_3 was blocked by heparin whereas it remained unaffected in the presence of ruthenium red. We were thus able to test the influence of UTP on the IP_3 receptors using heparin in the presence of ruthenium red. It appeared that 2.5 mg/ml heparin did not affect the UTP-induced tension while this concentration markedly reduced tensions induced by IP_3, suggesting that the UTP-induced Ca^{2+} release did not seem to involve the IP_3-sensitive channel.

Ca^{2+}-ATPase can mediate Ca^{2+} efflux from the sarcoplasmic reticulum in a coupled or reverse mode or in an uncoupling mode (De Meis, 1991). We tested these two alternative possibilities for the Ca^{2+} release stimulated by UTP. Some inhibitors of the sarcoplasmic reticulum ATPase like thapsigargin or cyclopiazonic acid are commonly used. However, thapsigargin has been demonstrated to inhibit both reverse function and uncoupled Ca^{2+}-ATPase (Du et al., 1996). Consequently, to determine whether the UTP-induced tension involved one of the two function modes of the Ca^{2+}-ATPase, we tested the ability of cyclopiazonic acid and compound 48/80 to inhibit the reverse function of the Ca^{2+}-pump based on previous experiments performed on vesicles (Du et al., 1996; Vale, 1990). We showed that compound 48/80 (100 μg/ml) abolished the UTP-induced tension. However, we did not obtain any inhibiting effect of cyclopiazonic acid on the UTP-induced tension. On the contrary, we observed activation of the Ca^{2+} release consistent with the leak efflux already noticed in presence of cyclopiazonic acid (Du et al., 1996). The cyclopiazonic acid-induced tension was insensitive to ryanodine but inhibited by compound 48/80. These data suggested that compound 48/80 was not specific to the reverse function of the Ca^{2+}-ATPase. The UTP induced tension thus might not involve this function mode of the Ca^{2+}-ATPase. The route of the leakage activated by cyclopiazonic acid and inhibited by compound 48/80 may correspond to the uncoupled Ca^{2+}-ATPase mode function.

We therefore considered the hypothesis of Ca^{2+} release through uncoupling of Ca^{2+}-ATPase which, in that case, operated as a channel. Previous studies have indicated that phenothiazines like trifluoperazine were able to stimulate this efflux of Ca^{2+} through the ATPase in the absence of P_i and ADP and that this efflux was antagonized by spermidine (De Meis, 1991). Our data demonstrated that 50 mM spermidine inhibited, in the same range, both the trifluoperazine- and UTP-induced tensions. These results suggested that the UTP-sensitive calcium release involved the uncoupled Ca^{2+}-ATPase. We also obtained inhibition of the cyclopiazonic acid-induced tension by spermidine, suggesting that cyclopiazonic acid may be an activator of this way of Ca^{2+} release. Activation of the Ca^{2+} efflux induced by trifluoperazine involved competition of this

drug with the P_i and Mg^{2+} binding sites of the enzyme, whereas spermidine blocked this efflux from interacting with regions that contained the Mg^{2+} and K^+ sites of the enzyme (De Meis, 1991). Previous experiments (Suarez-Kurtz et al., 1995) indicated that Ca^{2+} uptake by the sarcoplasmic reticulum was possible in the presence of UTP instead of ATP and that the UTP-induced release of sarcoplasmic reticulum-stored Ca^{2+} required Mg^{2+}, suggesting that MgUTP was the active form responsible for the enhanced release of Ca^{2+}. Since UTP seemed to act in the same way as trifluoperazine to induce Ca^{2+} release, we could hypothesize the existence of competition between UTP or rather MgUTP and the Mg^{2+} binding site of Ca^{2+}-ATPase. In conclusion, we suggest that a nucleotide such as UTP may activate calcium release from the sarcoplasmic reticulum through the uncoupled function mode of Ca^{2+}-ATPase.

Acknowledgements

This work was supported by a Research Grant from the Centre National d'Etudes Spatiales (CNES, no. 97/0292) and by grants from the Conseil Régional Nord Pas-de-Calais. The technical assistance of A.M. Lenfant is gratefully acknowledged.

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