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Intracellular calcium stores in β-escin skinned rat and guinea-pig bladders

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Abstract

Intracellular Ca^{2+} stores in rat and guinea-pig bladders and taenia caecum were studied in β-escin skinned smooth muscle strips. 30 min of skinning with 40 μM and 80 μM β-escin were the best parameters found to obtain good calcium response curves (10^{-7}–10^{-4} M) in rat and guinea pig, respectively. Calmodulin (1 μM) increased the calcium contractions significantly. pCa 6 was used to load intracellular stores and application of carbachol (50 μM) in all tissues then only contracted the tissues in the presence of guanosine-5′-triphosphate (GTP; 100 μM). Inositol triphosphate (IP_3; 50 μM), applied after pCa 6, contracted all tissues. Carbachol added after IP_3 or heparin (1 mg/ml) no longer caused a contraction in any of them. In bladders, caffeine (30 mM) but not ryanodine (5 μM) prevented the subsequent carbachol contraction. A slowly rising contraction with carbachol was elicited after caffeine (30 mM) or ryanodine (5 μM) in the taenia and after ryanodine in the bladders. Caffeine (30 mM) suppressed the calcium response curves in all tissues. Procaine (30 mM) blocked the carbachol (50 μM) contractions in bladders but not in taenia. These results suggest that calcium induced calcium release (CICR) and IP_3 induced calcium release (IICR) release calcium from a common store in bladder but two different compartments in taenia.

Keywords: β-escin skinned; Bladder; Taenia caecum; Intracellular calcium store; Carbachol

1. Introduction

Activation of the detrusor smooth muscle in rodents is through the parasympathetic nervous system, and involves the release of ATP activating P2X purinoceptors and acetylcholine activating M_3 muscarinic receptors. The purinergic system seems to be involved in small rapid contractions probably used in scent marking, and the cholinergic system to generate more prolonged contractions that empty the bladder during micturition (Brading and Mostwin, 1989). The mechanisms used by these two pathways to elevate intracellular calcium differ. The purinergic route probably involves initial calcium entry through the P2X receptors and through the voltage sensitive L-type calcium channels, as well as calcium release from stores via ryanodine receptors, and the cholinergic pathway is thought to involve inositol 1,4,5-trisphosphate (IP_3) dependent calcium release from internal stores. However the precise arrangement of the calcium stores in the detrusor and how they are utilized and interact is still currently not well defined, and there is controversy in the literature. It is becoming apparent that muscarinic receptor activation in the bladder may utilize several different mechanisms to initiate contraction, including rho kinase, although it is clear that IP_3 production and release of calcium from stores are involved (Iacovou et al., 1990; Wu et al., 2002).

A skinned smooth muscle, which is one having its plasma membrane permeabilized by a chemical agent, is a good pharmacological tool for investigating intracellular calcium events induced by agonists. β-escin is an agent that opens small holes in plasma membranes and therefore allows manipulation of intracellular ions and physiologically active substances. Compared to other skinning agents such as saponin and triton-X, β-escin has the advantage of leaving receptor–effector coupling intact. Hence, receptor mediated regulation of muscle contraction including intracellular signal transduction mechanisms can be studied, as well as the properties of IP_3 receptors, ryanodine receptors and calcium pumps. β-escin skinned smooth muscle, in contrast to intact muscle, is sensitive to calcium in the micromolar range (Satoh et al., 1999).

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Contraction in smooth muscle is initiated by an increase in cytoplasmic free calcium ($[Ca^{2+}]_i$). Electromechanical coupling occurs when the membrane is depolarized leading to calcium entry through voltage sensitive calcium channels. Inward calcium current during action potentials may also trigger calcium induced calcium release from the sarcoplasmic reticulum (Somlyo and Somlyo, 1994). Pharmacomechanical coupling following activation of G protein coupled receptors may also take place causing $[Ca^{2+}]_i$ increase by release from intracellular stores without a change in the membrane potential, and/or calcium sensitization (Somlyo and Somlyo, 1998). The two mechanisms of calcium release from the internal calcium stores, calcium induced calcium release (CICR) that is sensitive to ryanodine, and IP_3 induced calcium release (IICR) may interact in smooth muscles, and the calcium store is thought to consist of two compartments; *S alpha* having both CICR and IICR and *S beta* having only IICR, with different ratios in individual smooth muscles (Burdyga et al., 1995; Iino, 1990).

In this paper, we studied the properties of the intracellular Ca^{2+} stores using carbachol and activation of muscarinic receptors in rat and guinea-pig bladder, comparing the results with those obtained in strips of guinea-pig taenia caecum used as a control, since experiments on chemically skinned taenia have been published (Endo et al., 1977; Iino, 1989). The different properties of the stores in these tissues may reflect differences in tissue function as well as species differences.

2. Materials and methods

2.1. Tissue preparation

Male Wistar albino rats (150–200 g) and male guinea pigs (350–400 g) were stunned and bled to death as approved by the local ethics committee. The bladders from both species and taenia caecum from guinea pig were isolated and placed in Hepes buffered modified Krebs' solution. The mucosa and connective tissues were removed from the bladder under a dissecting microscope. Small strips (150–250 μm in diameter, 3–4 mm in length) were dissected. A snare was made to capture one end of the strip using 5/0 surgical silk and to mount it in a fixed position in a 500 μl chamber in one of a series of small chambers in a Perspex strip. The first chamber was filled with Hepes buffered modified Krebs' solution at room temperature and the strips were equilibrated for 30 min under a resting tension of 100 mg. Solution changes were made by moving the Perspex strips. The contractile force was measured by a sensitive force transducer (ADInstruments) connected to an Apple Macintosh PowerBook 1400c computer using Chart software and MacLab 8 hardware.

2.2. Drugs and solutions

All drugs and solutions were prepared in 18 MΩ-cm deionized water (Purelab UHQ, USF ELGA). Hepes buffered modified Krebs' solution contained (mM) NaCl 126; KCl 6; $CaCl_2$ 2; $MgCl_2$ 1.2; glucose 14 and HEPES 10.5. The pH of this Krebs' solution was adjusted to 7.2 with NaOH. 80 mM K^+ Krebs' solution was prepared by changing NaCl with an equivalent amount of KCl. Relaxing solution contained (mM) K propionate 130; $MgCl_2$ 4; Na_2ATP 4; tris–maleate 20; creatine phosphate 10; EGTA 4 and creatine phosphokinase 3.3 U/ml. The pH of this solution was adjusted to 6.8 with KOH. The drugs were always applied in relaxing solution with EGTA lowered to 0.05 mM. Activating solution was the same as relaxing solution except that EGTA was 10 mM and calcium was added. The free calcium concentration was changed by adding an appropriate amount of $CaCl_2$ to activating solution, calculated using a computer program ('Bound and Determined', (Brooks and Storey, 1992)) and expressed as the negative logarithm (pCa). Protease inhibitor leupeptin (1 μM) and mitochondrial blocker FCCP (1 μM) were also added to the activating solution. Calmodulin (1 μM) was present in all the experiments except the control and caffeine groups in cumulative calcium response curves.

Drugs used were β-escin (aescin), carbamylcholine chloride (charbachol), caffeine, creatine phosphokinase, leupeptin, ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraasetic acid (EGTA), adenosine 5′-triphosphate (Na_2ATP), ryanodine, guanosine-5′-triphosphate (GTP), calmodulin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), dimethylsulphoxide (DMSO) from Sigma (St. Louis, Missouri); creatine phosphate disodium salt from Calbiochem (Nottingham, United Kingdom); procaine HCl from BDH.

2.3. Experimental procedures

2.3.1. pCa tension curves

After stable responses had been achieved to 80 mM K^+ and 50 μM carbachol in intact strips, strips were moved into relaxing solutions, incubated for a few minutes and then permeabilized with 40 μM and 80 μM β-escin in relaxing solution (composition given above) for rat and guinea pig respectively at pH 6.8. Muscle fibers were accepted as skinned if the maximum tension obtainable by 10^{-4} M calcium in the presence of 1 μM calmodulin after skinning was found to be greater than the tension produced by 80 mM K^+ applied in the same strip before the skinning procedure (Endo et al., 1977).

After the skinning procedure, the preparations were relaxed by exposure to the relaxing solution for 4 min. Increasing concentrations of calcium were applied cumulatively in activating solution to elicit calcium response curves. The effects of calmodulin and caffeine on calcium response curves were obtained in unpaired tissues, since after skinning the second response curve decreased compared to the first one.

2.3.2. Store release experiments

Intracellular calcium stores were loaded by incubating the skinned tissues in activating solution at pCa 6 for 10 min, followed by a wash in relaxing solution until the tonus returned to base line. Stored calcium was then assessed by inducing a contraction with carbachol (50 μM) in relaxing solution containing GTP (100 μM) to replenish the loss of endogenous GTP (Kitazawa et al., 1989).

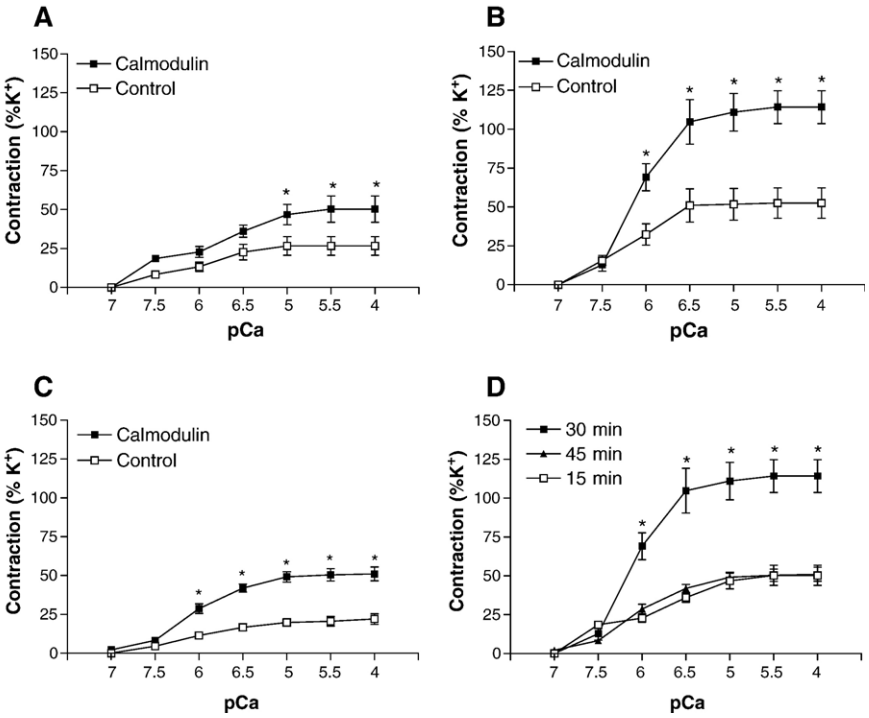

Fig. 1. Calcium response curves (pCa 7 to pCa 4) in the absence (□) and presence (■) of 1 μM calmodulin in (A) 15 min, (B) 30 min and (C) 45 min β-escin skinned rat bladder. In all three groups calmodulin (■) increased the contractions significantly compared to their own controls (□) (n=4–5, *P<0.05, ANOVA). (D) is the summary showing calcium response curves (pCa 7 to pCa 4) in the presence of 1 μM calmodulin after 15 min (□), 30 min (■) and 45 min (▲) of skinning. Contractions elicited after 30 min was significantly different from both 15 min and 45 min of skinning (n=4–5, *P<0.05, ANOVA).

2.4. Data analysis

Contractions are expressed as % of the response to 80 mM K^+ elicited in intact tissues before skinning. Data are given as mean±S.E.M. of n experiments. Statistical analysis was done using one way analysis of variance (ANOVA) followed by Bonferroni test for comparing multiple groups. Student's t-test was used for comparing two groups. $P<0.05$ was accepted as statistically significant.

3. Results

3.1. Calcium response curves and the effect of calmodulin in skinned preparations

80 mM K^+ applied to intact tissues initiated contractions that were 142.6±12.51, 168.3±11.28 and 245.2±13.81 mg in rat bladder (n=54), guinea-pig bladder (n=54) and taenia caecum (n=56), respectively.

In order to estimate the best conditions to obtain viable preparations, we varied the concentration of β-escin used for skinning, and the time of exposure, before obtaining calcium response curves with cumulative addition of calcium (pCa 7–pCa 4). In the rat, best results were obtained using 40 μM β-escin, and in the guinea-pig tissues with 80 μM β-escin. Cumulative calcium response curves were then obtained in the absence and presence of calmodulin (1 μM).

Fig. 1A–C illustrates experiments carried out in rat bladder strips following 15, 30 and 45 min skinning with 40 μM β-

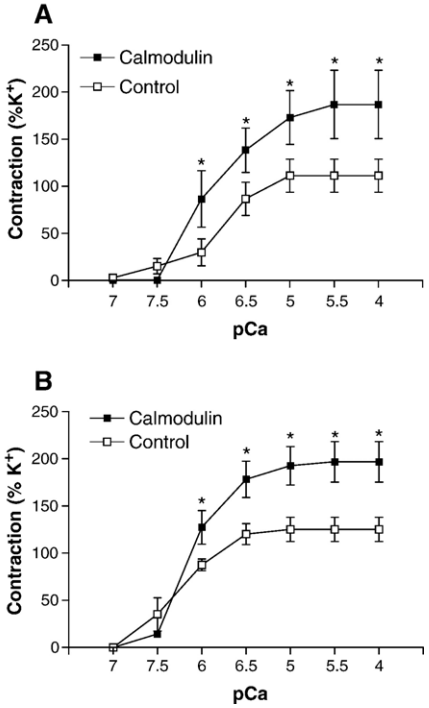

Fig. 2. Calcium response curves (pCa 7 to pCa 4) in the absence (□) and presence of 1 μM calmodulin (■) in 30 min β-escin skinned (A) guinea-pig bladder and (B) guinea-pig taenia. In both groups calmodulin (■) increased the contractile responses significantly compared to control (□) (n=4–6, *P<0.05, ANOVA).

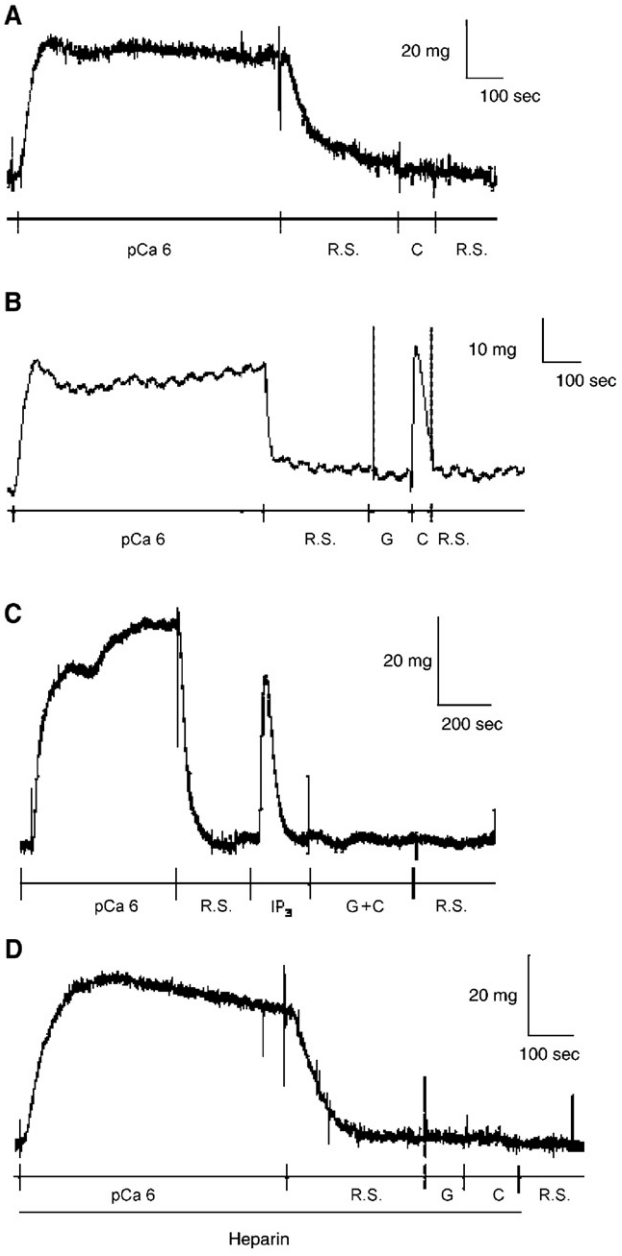

Fig. 3. The effects of GTP, IP_3 and heparin on carbachol-induced contractions in guinea-pig bladder strips. To load the stores the strips were exposed to pCa 6 for 10 min and then placed in relaxing solution containing 4 mM EGTA but no calcium for approximately 4 min. (A) Carbachol (C; 50 μM) alone failed to evoke contraction ($n=6$). (B) Application of GTP (G; 100 μM) for approximately 1 min allowed the following carbachol application (CCh, 50 μM) to induce contraction ($n=4$). (C) IP_3 (50 μM) caused contraction, and blocked the subsequent response to carbachol ($n=6$). (D) Heparin (1 mg/ml) applied throughout also blocked the response to carbachol ($n=4$).

escin. In each group the maximum contraction elicited with pCa 4 was seen after a 30 min skinning (52.5±9.7%). This value was significantly greater than that after 15 and 45 min skinning (26.7±5.9 and 22.0±3.4%, respectively; $P<0.05$). Calmodulin (1 μM) caused a statistically significant increase in calcium response curves in each group compared to control curves ($P<0.05$), implying the loss of endogenous calmodulin in β-escin skinning. Fig. 1D, is a summary, comparing the calcium response curves in the presence of calmodulin after different skinning times, and showing that the responses after 30 min β-escin skinning grew to 114.3±10.6% and were significantly greater than after 15 min and 45 min skinning ($P<0.05$).

The best responses were obtained from guinea-pig bladder and taenia caecum after 30 min skinning with 80 μM β-escin. The maximum contractions elicited by pCa 4 in both tissues were greater than 80 mM K^+ contractions before skinning in the absence of calmodulin; 111.3±17.5% in bladder and 125.2±12.8% in taenia caecum. Calmodulin (1 μM) increased the contractions significantly both in bladder and in taenia caecum compared to their own controls ($P<0.05$); increasing the maximum contractions to 186.9±36.4% in bladder and 196.9±21.5% in taenia caecum (Fig. 2A and B).

3.2. The effect of GTP on carbachol contractions after calcium loading in skinned smooth muscles

Application of pCa 6 for 10 min to load intracellular calcium stores in β-escin skinned strips caused contractions that were

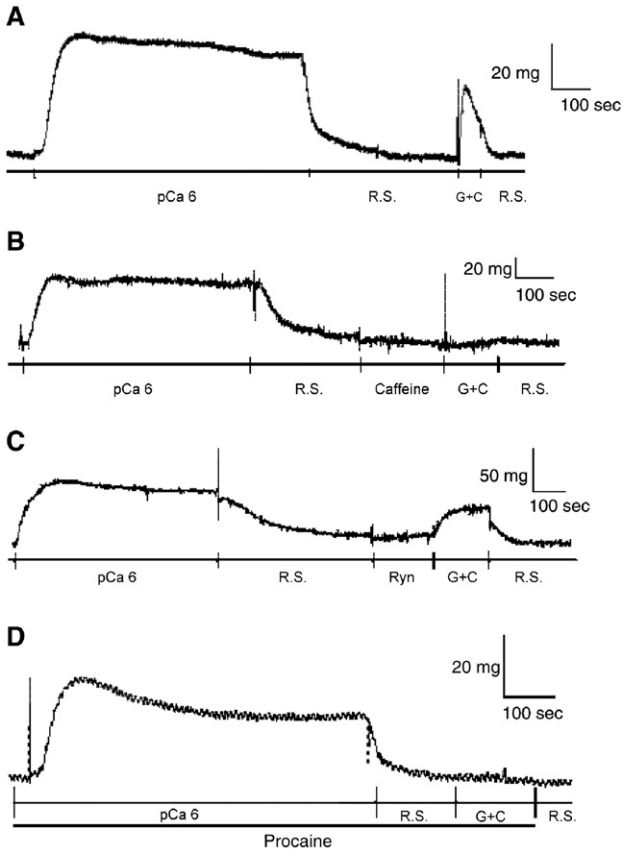

Fig. 4. The effect of caffeine and ryanodine on carbachol contractions in rat bladder. To load the stores the strips were exposed to pCa 6 for 10 min and then placed in relaxing solution containing 4 mM EGTA but no calcium for approximately 4 min. (A) Subsequent application of GTP (G; 100 μM) and carbachol (C, 50 μM) induced a contraction ($n=6$). (B) Caffeine (30 mM) applied for approximately 2 min prevented the contraction ($n=4$). (C) Ryanodine (5 μM) applied for 2 min abolished the phasic response to carbachol but revealed a slowly developing contraction ($n=6$). (D) In the presence of procaine (30 mM), GTP (G; 100 μM) and carbachol (C, 50 μM) caused no contraction ($n=5$).

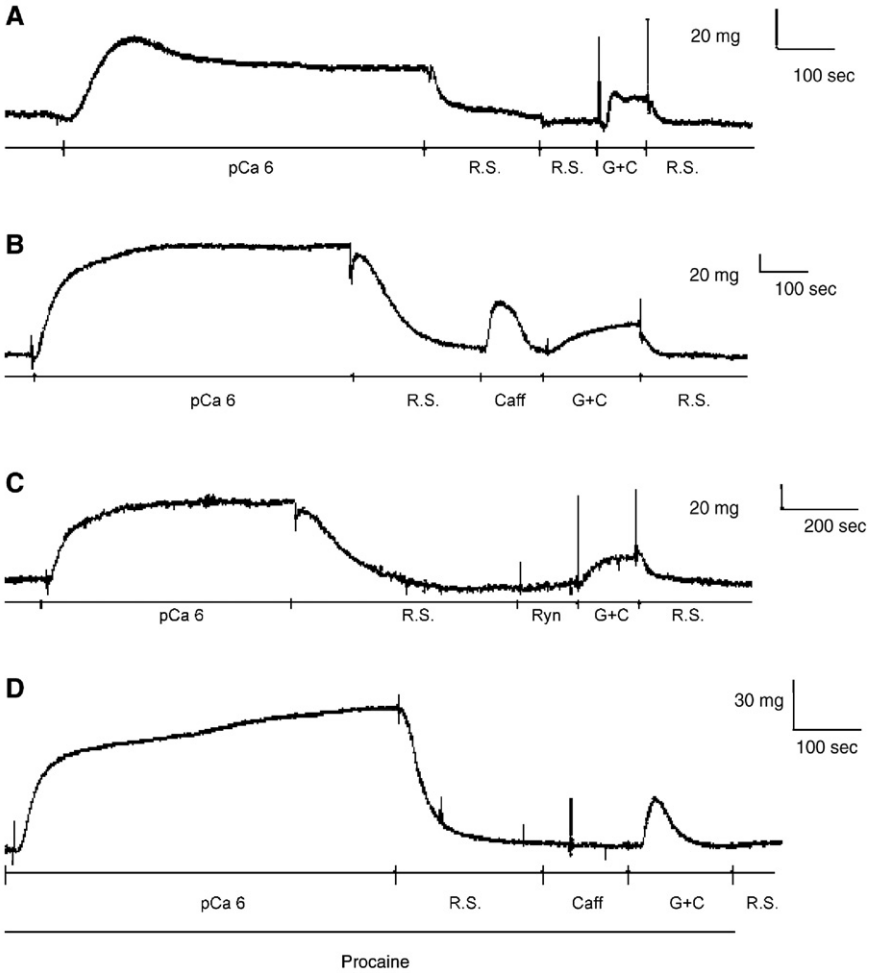

Fig. 5. The effect of caffeine, ryanodine and procaine on carbachol contractions in guinea-pig taenia caecum. The strips were first exposed to pCa 6 for 10 min and then to relaxing solution having 4 mM EGTA but no calcium for approximately 4 min. (A) Application of GTP (G; 100 μM) and carbachol (C, 50 μM) induced a contraction (n=4). (B) Caffeine (30 mM) applied for approximately 2 min before GTP and carbachol-induced a contraction itself and abolished the phasic response to carbachol allowing a slowly developing contraction (n=4). (C) Ryanodine (5 μM) applied for 2 min before GTP and carbachol did not induce a contraction, but it abolished the phasic response to carbachol allowing a slowly developing contraction (n=4). (D) In the continuous presence of procaine (30 mM) caffeine no longer contracted the strip, but the subsequent application of GTP (G; 100 μM) and carbachol (C, 50 μM) now produced a phasic contraction (n=4).

respectively 86.6±3.0, 69.1±1.9 and 126.9±7.3% in guinea-pig bladder, rat bladder and guinea-pig taenia strips (n=4–6). After the tissues had been returned to the relaxing solution, carbachol (50 μM) alone did not cause any contraction (e.g. Fig. 3A). However, if GTP (100 μM) was applied for approximately 1 min, carbachol (50 μM) then initiated a contraction that was respectively 36.4±5.1, 33.6±5.9 and 30±3.2% in guinea-pig bladder, rat bladder and guinea-pig taenia strips (n=4–6) as shown in Figs. 3B, 4A and 5A. This suggests that GTP is lost from the skinned tissues, and is necessary for the carbachol response.

3.3. The effects of IP_3 and heparin

The intracellular second messenger IP_3 (50 μM) applied after loading the intracellular stores with pCa 6 caused a contraction in all three tissues which was 51.9±5.1, 65.0±6.7, 70.9±6.5% for rat bladder, guinea-pig bladder and taenia caecum, respectively (n=4–5). Carbachol (50 μM) in the presence of GTP (100 μM) added after IP_3 was no longer able to cause a contraction in any of the tissues (Fig. 3C). The IP_3 receptor inhibitor heparin (1 mg/ml) applied in all solutions after β-escin skinning also abolished the contractile response to carbachol in all three tissues (n=4) as shown in guinea-pig bladder in Fig. 3D. These results support the suggestion that carbachol under these experimental procedures does indeed induce an IP_3 dependent release of calcium from the sarcoplasmic reticulum.

3.4. The effects of caffeine, ryanodine and procaine

In order to evaluate further the nature of the intracellular calcium stores in these three tissues, we investigate the effects of caffeine, ryanodine and procaine which have different mechanisms of action on the stores. Caffeine was used to empty stores containing ryanodine receptors through activation of CICR and procaine was used to block CICR. Ryanodine, which is thought to either open or block the ryanodine receptors depending on the concentration, was used in high concentration to block these receptors (Itoh et al., 1981).

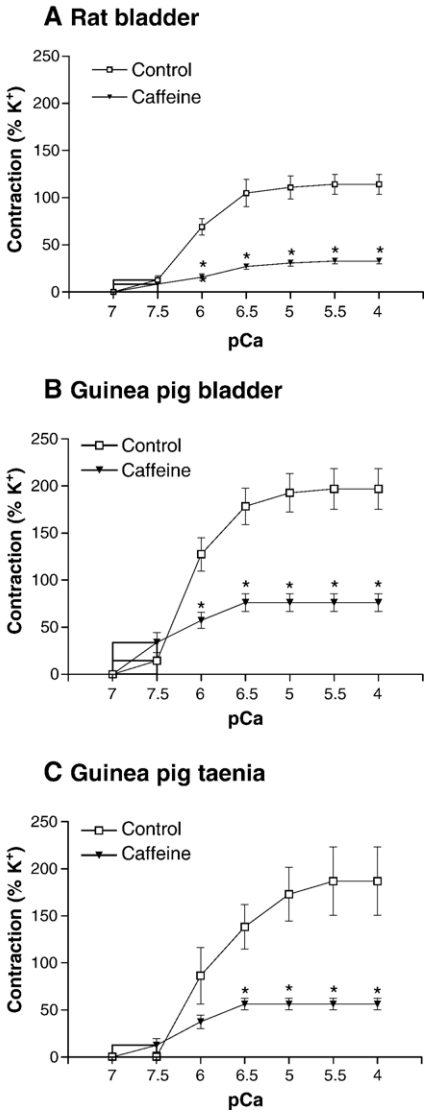

Fig. 6. Calcium response curves (pCa 7 to pCa 4) in the absence (□) and presence of 30 mM caffeine (▼) in 30 min β-escin skinned (A) rat bladder, (B) guinea-pig bladder and (C) guinea-pig taenia. In both groups caffeine (▼) decreased the contractile responses significantly compared to control (□) (n=4–6, *P<0.05, ANOVA). All contractions were elicited in the presence of calmodulin.

In rat bladder strips caffeine (30 mM) (applied for approximately 2 min before GTP and carbachol) did not cause contraction or relaxation of the tissue but prevented the subsequent contraction by carbachol (n=4; Fig. 4B). Ryanodine (5 μM) was also tried in the same protocol. As shown in Fig. 4C, ryanodine also had no effect on its own, but in contrast to caffeine, the response to carbachol was still present (25.7±3.4% after ryanodine v. 33.6±5.9% control, n=6; P>0.05), but converted from a phasic to a slowly rising contraction. Similar results were found in guinea-pig bladder (31.9±6.8% after ryanodine v. 36.4±5.1% control, n=6, P>0.05). The inability of ryanodine to inhibit the carbachol response could be interpreted to suggest that CICR stores are not involved in this response, but the ability of caffeine to suppress carbachol responses is not consistent with this interpretation.

Since in many smooth muscles caffeine will contract tissues while emptying the stores, we repeated these experiments on strips of guinea-pig taenia. In this tissue caffeine (30 mM), applied after loading the stores at pCa 6, transiently contracted the strips (36.0±7.1%, n=4, Fig. 5A). After caffeine treatment, subsequent application of GTP and carbachol no longer caused a phasic contraction, but a slowly rising contraction to 40.5±9.4% (n=4) was elicited (Fig. 5B). Ryanodine (5 μM) applied for 2 min did not affect the tissue itself but like caffeine, it abolished the rapid phasic response to carbachol, while leaving a slowly developing contraction to 29.2±9.1% (v. 30±3.2% control, n=4; P>0.05, Fig. 5C).

Since caffeine is known to have the ability to block phosphodiesterases, and through this mechanism can relax smooth muscles, we investigated the effects of caffeine further, looking at caffeine's effects on the calcium response curves. In all three tissues, calcium response curves were suppressed significantly (P<0.05) if the experiments were carried out in the presence of caffeine (30 mM) suggesting an inhibitory role of caffeine on the contractile mechanisms in β-escin skinned smooth muscles (Fig. 6A, B and C).

In an attempt to clarify the situation we have examined the effects of procaine to block CICR. In rat and guinea-pig bladder, incubation with procaine (30 mM) inhibited the carbachol contraction as shown in Fig. 4D for rat. This suggests that CICR and IICR release calcium from a common calcium store in bladder smooth muscle. In guinea-pig taenia caecum, however, procaine (30 mM) inhibited the contractile response evoked by caffeine (30 mM) but did not affect the subsequent carbachol (50 μM) contraction elicited in the presence of GTP (100 μM) (33.8±4.0%, not significantly different from the control i.e. 30±3.2%, P>0.05) suggesting that in this tissue there are two different compartments of the sarcoplasmic reticulum (Fig. 5D; n=4).

4. Discussion

The results in this paper demonstrate differences in the properties of calcium release from intracellular stores between bladder and taenia caecum smooth muscles. The properties of the three tissues are summarised in Table 1. It is interesting that the bladder stores seem similar in the rat and guinea-pig, which is in contrast to the stores in the ureter, that show markedly different species dependent properties (Burdyga et al., 1995).

Skinned preparations of rat and guinea-pig bladders and of taenia caecum, could be activated by cumulative addition of 10^{-7}–10^{-4} M calcium to the organ bath. Since it has been shown in many other smooth muscles that skinning with β-escin leads to loss of some endogenous substances such as calmodulin and GTP (Litosch and Fain, 1986; Loirand et al., 1999; Todoroki-Ikeda et al., 2000), adjusting the time and concentration of the chemical agent used and replacing the lost endogenous substances play a major role in successful skinning of smooth muscle strips.

We tried three different incubation times in rat detrusor muscle at constant β-escin concentration (40 μM) to find the most suitable parameters. After both 15 min and 45 min incubation

Table 1
Summary of the effects of different agents on rat bladder, guinea-pig bladder and guinea-pig taenia caecum (CaM, calmodulin; CCh, carbachol)

Agents	Rat bladder	Guinea-pig bladder	Guinea-pig taenia
β-escin	40 μM for 30 min	80 μM for 30 min	80 μM for 30 min
CaM	Increased the Ca^{2+} response curve	Increased the Ca^{2+} response curve	Increased the Ca^{2+} response curve
CCh	Contracted only in the presence of GTP	Contracted only in the presence of GTP	Contracted only in the presence of GTP
IP_3	Contracted	Contracted	Contracted
Heparin	Blocked CCh contraction	Blocked CCh contraction	Blocked CCh contraction
Caffeine	Decreased the Ca^{2+} response curve	Decreased the Ca^{2+} response curve	Decreased the Ca^{2+} response curve
Caffeine	Blocked the subsequent CCh+GTP contraction	Blocked the subsequent CCh+GTP contraction	Contracted itself and caused a slowly rising CCh contraction
Ryanodine	Caused a slowly rising CCh contraction	Caused a slowly rising CCh contraction	Caused a slowly rising CCh contraction
Procaine	Blocked CCh contraction	Blocked CCh contraction	Blocked caffeine contraction but not the subsequent CCh contraction

with β-escin in rat bladder the smooth muscle strips contracted less than they did after 30 min skinning (Fig. 1A,B,C), probably due to insufficient skinning and loss of calmodulin respectively. The largest contractions in the calcium response curve were observed after 30 min skinning and this was accepted as the right parameter and used in future experiments. In guinea pig, however, 40 μM β-escin skinning for 30 min was found insufficient and 80 μM skinning for 30 min was accepted for both guinea-pig bladder and taenia caecum. In all tissues and each timing protocol, addition of calmodulin increased the contractions significantly proving the loss of endogenous calmodulin.

Results in this study (Fig. 3A and B for guinea-pig bladder) and in our previous paper (Durlu-Kandilci and Brading, 2006) showed that in smooth muscle skinned with β-escin, exogenous GTP is necessary to elicit a contraction by the muscarinic agonist carbachol. These findings parallel the work by Kobayashi et al. in β-escin skinned guinea-pig ileum (Kobayashi et al., 1989).

Muscarinic receptor activation in the bladder is normally thought to induce contraction mainly through IP_3 production and release of calcium from internal stores (Iacovou et al., 1990) although there is a clear involvement of calcium entry through L-type calcium channels and CICR dependent stores (Mostwin, 1985; Wu and Fry, 1998; Wu et al., 2002; Rivera and Brading, 2006). In all three tissues, exogenous IP_3 caused a contraction and subsequent application of carbachol did not cause any response showing that the contractile response initiated by carbachol does require calcium release from an IP_3 sensitive store. The IP_3 receptor inhibitor heparin also abolished the contractile response to carbachol in all three tissues supporting this postulation (Fig. 3D for guinea-pig bladder).

In this study, caffeine contracted the taenia, but not the rat or guinea-pig detrusor. When it was applied before GTP and carbachol, it converted the rapid carbachol contraction into a small slowly developing one in the taenia, but prevented the subsequent carbachol contraction in both rat and guinea-pig bladders (Fig. 4B for rat bladder). The inhibitory effects of caffeine are well known. In smooth muscle of rat aorta, caffeine lowered the $[Ca^{+2}]_i$ and the sensitivity of contractile mechanisms to calcium (Sato et al., 1988). In pancreatic acinar cells, caffeine has been observed to block IP_3 production and calcium release by IP_3, thus inhibiting acetylcholine responses (Toescu et al., 1992). More importantly, due to its chemical structure as a methylxanthine, caffeine is known to inhibit cyclic nucleotide phosphodiesterase and increase cyclic adenosine monophosphate levels (Butcher and Sutherland, 1962; Belibi et al., 2002; Lindaman et al., 2002). In rat bladder, phosphodiesterase inhibitors are known to relax previously contracted strips (Qiu et al., 2001). In mouse bladder smooth muscle, caffeine did not cause any contraction but suppressed carbachol contractions (Sugita et al., 1998). We think it important to emphasize that in bladder but not in taenia, the contraction suppressing effect of caffeine as a phosphodiesterase inhibitor is more potent than its contractile effect as a CICR activator. Thus, the inability of caffeine to contract the detrusor cannot be used as evidence to suggest that ryanodine receptors are not involved in the muscarinic response.

Ryanodine, used in a concentration that inhibits calcium release from CICR, had no contractile effect on its own on any of the skinned tissues. Unlike caffeine, however, it did not entirely inhibit the carbachol contractions in any of the tissues, but converted the fast contractile responses into slowly rising contractions. This ability of ryanodine suggests that CICR stores are involved in this carbachol response. Procaine on the other hand, blocked the response to carbachol in bladders but not in taenia caecum. This again emphasizes the contribution of ryanodine receptors to muscarinic receptor activation in detrusor smooth muscle.

It is interesting to speculate about the differences between the tissues with respect to stores. Why does caffeine not contract detrusor but does contract taenia? Why does ryanodine in all three muscles and caffeine in the taenia convert the rapid contractile response to carbachol into a more slowly rising contraction? Electron micrographs show that the sarcoplasmic reticulum in all three tissues exists predominantly close to the plasma membrane in association with membrane caveolae, although some central elements are also seen particularly in the taenia, and it is therefore unlikely that differences in the location will account for the different properties (Gabella and Uvelius, 1990; Moore et al., 2004; Gherghiceanu and Popescu, 2006). It is interesting too that in the skinned bladders, the rapid contraction to carbachol before ryanodine is larger in amplitude than the slow contraction, whereas in the skinned taenia, there is a much smaller rapid contraction to carbachol, and the slowly rising contraction is about the same size. These results suggest that calcium release from the stores in taenia is more readily activated through ryanodine receptors than in the bladder. Thus caffeine alone can release enough calcium in the taenia to contract in spite of any inhibitory effects through the rise in cyclic nucleotides. Also this could account for the much smaller

size of the carbachol response in the skinned taenia, since there would normally be a substantial contribution of calcium entry from the extracellular space, as muscarinic receptor activation causes marked depolarization of the intact taenia (Bulbring and Kuriyama, 1963), whereas only a small depolarization occurs in the bladder (Fujii, 1988).

In the detrusor, the dominant release mechanism is probably through IP_3 receptors, but the ability of ryanodine to reduce and slow the carbachol contraction can be best interpreted by suggesting that muscarinic receptor activation results in some activation of calcium entry, and CICR dependent calcium release (Brading and Inoue, 1991). One could postulate that this calcium can then sensitize the IP_3 receptors, to cause an IP_3-dependent rapid release of calcium from the stores. Pretreatment with ryanodine to suppress CICR would abolish this initial step, and the IP_3-dependent release from the stores under these conditions might develop more slowly, accounting for the slow contractile response.

There are also other mechanisms involved in the carbachol response of the detrusor and taenia as we have previously shown but, these rho kinase and protein kinase C pathways are particularly important in calcium sensitization caused by receptor activation and can be observed when intracellular calcium is held constant (Durlu-Kandilci and Brading, 2006). In the present study, as shown from the caffeine and procaine results, the intracellular stores of the bladders from different species have similar properties, whereas if carbachol-induced calcium sensitization, a phenomenon not related to IP_3 receptors, is taken into consideration (Durlu-Kandilci and Brading, 2006), differences between rat and guinea-pig detrusor become evident.

In conclusion, contractile responses after skinning with β-escin require both exogenous calmodulin and GTP in all three tissues. The different behaviour of the tissues with respect to store release suggests that the there are two distinct stores in the taenia, since caffeine does not completely block the muscarinic response. In the bladders, IP_3 and CICR probably release calcium from an overlapping store, although the overall inhibitory effect of caffeine makes firm conclusions difficult. More calcium seems to be released using CICR in the muscarinic response of the taenia than the bladder. In the bladder, the IP_3 receptors may be responsible for muscarinic receptor mediated calcium release leading to contraction, but CICR may play an important role in elevating calcium concentrations locally and sensitizing the IP_3 receptors to allow rapid release of calcium. CICR is also used to elevate calcium in the purinergic response to nerve stimulation.

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