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Endothelin B receptor Ca^{2+} signaling in shark vascular smooth muscle: participation of inositol trisphosphate and ryanodine receptors

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Summary

In mammals, endothelin receptors are sub-classified into ET_A receptors (ET_AR), which are purely constrictive in vascular smooth muscle (VSM), and ET_BR, which may produce constriction in VSM or dilatation by stimulating the production of nitric oxide (NO) from endothelial cells. In contrast, previous studies suggested that shark VSM is stimulated exclusively by ET_BR. The Ca^{2+} signaling pathways utilized by shark VSM in response to stimulation by endothelin-1 (ET-1) have not previously been investigated. We measured cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2-loaded VSM of anterior mesenteric artery of *Squalus acanthias* and show that the ET_BR agonists IRL 1620 and sarafotoxin S6c (SRX) increase $[Ca^{2+}]_i$ in VSM to the same extent as ET-1 and ET_BR appears to be the only ETR subtype in sharks. To investigate the participation of the inositol trisphosphate (IP_3) receptors (IP_3R), we utilized two inhibitors of the mammalian IP_3R, TMB-8 and 2-APB. In Ca^{2+}-free Ringer, these agents inhibit the response to ET_BR agonist stimulation by 71%. The ryanodine-sensitive receptor (RyR) may be activated by low concentrations of ryanodine, by abrupt local increases of $[Ca^{2+}]_i$, (calcium-induced calcium release) or by cyclic adeninediphosphate ribose (cADPR). We employed three inhibitors of activation of the RyR, Ruthenium Red, 8-Br cADPR and high concentrations of ryanodine; these agents blocked the $[Ca^{2+}]_i$ response to ET_BR agonist stimulation by a mean of 39%. These data show for the first time that in VSM of the shark, ET_BR activation stimulates both IP_3R and RyR, and that cADPR is involved in RyR activation.

Key words: calcium signaling, elasmobranch, cADPR, ryanodine, IP3, dogfish shark, Squalus acanthias.

Introduction

The mechanisms by which agonist stimulation of vascular smooth muscle (VSM) in *Squalus acanthias* increases cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) to initiate contraction have not been studied. Previously we presented evidence for activity of a ryanodine-sensitive receptor (RyR) and an inositol trisphophate (IP_3) receptor (IP_3R) in VSM of the rectal gland artery of *S. acanthias* (Fellner and Parker, 2002). We noted activation of the IP_3R following stimulation of a polycationionic sensing receptor with Ca^{2+}, spermine or gadolinium, as has been shown in mammalian tissues (Conigrave et al., 2000). A RyR had previously been demonstrated in dogfish ventricles (Thomas et al., 1996), but its function in shark VSM has not been investigated.

Endothelin is considered to be among the most potent initiators of vascular contraction in mammalian species (Yanagisawa et al., 1988); its receptors are sub-classified into ET_AR and ET_BR. In mammals, activation of the ET_AR on VSM is purely constrictive, whereas stimulation of ET_BR may cause either dilatation, by promoting the release of nitric oxide (NO) and sometimes prostacyclin from endothelial cells, or constriction of VSM (D'Orleans-Juste et al., 2002). In *S. acanthias*, VSM of the ventral aorta and anterior mesenteric artery appears to respond to ET_BR but not ET_AR agonist stimulation (Evans, 2001). The ET_BR-specific agonist sarafotoxin S6c (SRX) was as constrictive as endothelin-1 (ET-1) to arterial rings.

In mammals, ET-1 has been shown to mobilize Ca^{2+} from the endoplasmic (ER) or sarcoplasmic reticulum (SR) *via* activation of both the IP_3R and RyR (Shimoda et al., 2000). Cyclic ADP-ribose (cADPR) is synthesized from nicotinamide adenine dinucleotide (NAD) following ET-1 stimulated ADP-ribosyl cyclase activity. Then cADPR activates the RyR, causing a release of Ca^{2+} from the endoplasmic or sarcoplasmic reticulum. Involvement of cADPR in ET-1 induced contraction has been demonstrated in rat mesenteric small arteries (Giulumian et al., 2000). However, whether both ET_AR and ET_BR activation signals involve both IP_3R and RyR activation in VSM is unknown. In rat peritubular smooth muscle of seminiferous tubules, ET_AR activation involves both IP_3R and RyR whereas ET_BR works exclusively through the

RyR *via* formation of cADPR (Barone et al., 2002). That the dogfish shark appears to have only ET_BR on VSM of the aorta and anterior mesenteric artery raises the question of whether ET_BR signals through both IP_3R and RyR or exclusively through RyR. Thus investigating the participation of these SR receptors in shark may answer important questions applicable to all vertebrate VSM. We have utilized the specific ET_BR agonists IRL-1620 and SRX to explore the involvement of IP_3R and RyR activation in the anterior mesenteric artery of *S. acanthias*.

Materials and methods

Sharks *Squalus acanthias* L. (2–3 kg) of both sexes were caught in Frenchman Bay, Maine, USA, and kept in running seawater tanks (11–15°C early to late summer) until the animals were pithed through the snout and killed. This protocol was approved by the Institutional Animal Care Committee at Mount Desert Island Biological Laboratory, Salisbury Cove, ME, USA.

The anterior mesenteric artery was dissected and placed in ice-cold Ca^{2+}-free shark Ringer (pH 7.7) containing, in mmol l^{-1}, NaCl, 275; KCl, 4; $MgCl_2$, 3; Na_2SO4, 0.5; KH_2PO_4, 1.0; $NaHCO_3$, 8; urea, 350; D-glucose, 5; and trimethylamine oxide (TMAO), 72 (Fellner and Parker, 2002). The complete buffer contained 2.5 mmol l^{-1} calcium (normal concentration in the shark), whereas no $CaCl_2$ was added to the nominally calcium-free buffer. Unless otherwise specified, the Ca^{2+} concentration in the buffer was 2.5 mmol l^{-1}. The anterior mesenteric artery was minced into pieces <0.5 mm in size and then loaded with the Ca^{2+}-sensitive fluorescent dye, fura-2AM. Cytosolic $[Ca^{2+}]_i$ was measured as previously described (Fellner and Arendshorst, 2000, 2002; Fellner and Parker, 2002). Arterial tissue was placed in an open static chamber and examined in a small window of the optical field of a ×40 oil-immersion fluorescence objective of an inverted microscope (Olympus IX70, Tokyo, Japan). Approximately 5–6 typical elongated vascular smooth muscle cells were selected for analysis. The tissue was excited alternately with light of 340 and 380 nm wavelengths from a dual-excitation wavelength Delta-Scan equipped with dual monochronometers and a chopper (Photon Technology International, NJ, USA). After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. The calibration of $[Ca^{2+}]_i$ was based on the signal ratio at 340/380 nm and known concentrations of Ca^{2+} (Grynkiewicz et al., 1985) and was performed prior to initiating the experimental protocol.

The concentrations of ET-1, IRL 1620 and SRX that we employed in each experiment were 2×10^{-7} mol l^{-1}, a concentration at least twice the maximal stimulatory concentrations reported in the literature (Just et al., 2004; Touyz et al., 1995; Shimoda et al., 2000; Yanagisawa et al., 1988; Cavarape et al., 2003; Batra et al., 1993). The concentrations of antagonists were at least tenfold greater than that of the agonists and were also chosen on the basis of values reported in the literature.

Reagents

TMAO, Ruthenium Red, 8-Br cADPR, 8-(*N*,*N*-diethylamino) octyl 3,4,5-trimethoxybenzoate (TMB-8) and 2-aminoethyl diphenyl borate (2-APB) were purchased from Sigma (St Louis, MO, USA), ET-1 and IRL-1620 from California Peptide Research (Napa, CA, USA), ryanodine from Calbiochem (San Diego, CA, USA), sarafotoxin S6c from American Peptide (Sunnyvale, CA, USA) and fura-2AM from Teflab (Austin, TX, USA).

Statistics

The data are presented as means ± standard error of the mean (S.E.M.). Each data set is derived from tissue originating from at least three different sharks. Paired data sets were tested using Student's paired *t*-test. Multiple comparisons were analyzed using one-way analysis of variance (ANOVA) for repeated measures followed by Student–Neuman–Kuels *post hoc* test. $P<0.05$ was considered statistically significant.

Results

Response to IRL-1620, sarafotoxin S6c and endothelin-1 in Ca^{2+}-free Ringer

Endothelin is a ligand for both the ET_AR and ET_BR. There are a number of specific agonists for the ET_BR but, to date, no specific agonist has been found for the ET_AR. To confirm that the anterior mesenteric artery of the dogfish shark responds to ET_BR but not ET_AR stimulation, we measured the $[Ca^{2+}]_i$ response to two specific ET_BR agonists, IRL 1620 and SRX, in Ca^{2+}-free Ringer. IRL 1620 increased $[Ca^{2+}]_i$ by 89±26 nmol l^{-1} (N=19, $P<0.01$ compared to baseline); the increase in $[Ca^{2+}]_i$ following SRX was 76±11 nmol l^{-1}, which was not different from the response produced by IRL 1620 (N=10, P=0.34 for IRL *vs* SRX). ET-1 alone increased $[Ca^{2+}]_i$ by 79±15 (N=6, $P<0.05$ *vs* IRL 1620 or SRX), demonstrating that ET-1, a combined ET_AR and ET_BR agonist had the same effect on increasing $[Ca^{2+}]_i$ in VSM as the selective ET_BR agonists, IRL 1620 and SRX (Fig. 1A).

In VSM known to have both ET_AR and ET_BR, stimulation first by IRL 1620 produces a peak response similar to that in shark mesenteric artery; however, subsequent addition of ET-1 causes a second peak, which is about twice the magnitude of the IRL 1620-induced peak (Fellner and Arendshorst, 2004), showing that initial application of ET_BR agonists does not cause ET_AR desensitization, at least in rat VSM. It is conceivable that substantial differences exist between mammalian and fish VSM. To further demonstrate that ET-1 activates ET_BR but not ET_AR in shark VSM, we treated the VSM with IRL 1620; at the nadir of the response we added ET-1. Fig. 1B, a representative tracing of the response of mesenteric artery VSM to IRL 1620, illustrates the typical pattern in the shark mesenteric artery VSM of a rise to a peak over a 10–20 s period and an unusually long peak duration of nearly 100 s. When ET-1 was added at the nadir of the $[Ca^{2+}]_i$ response to IRL 1620, there was no further significant

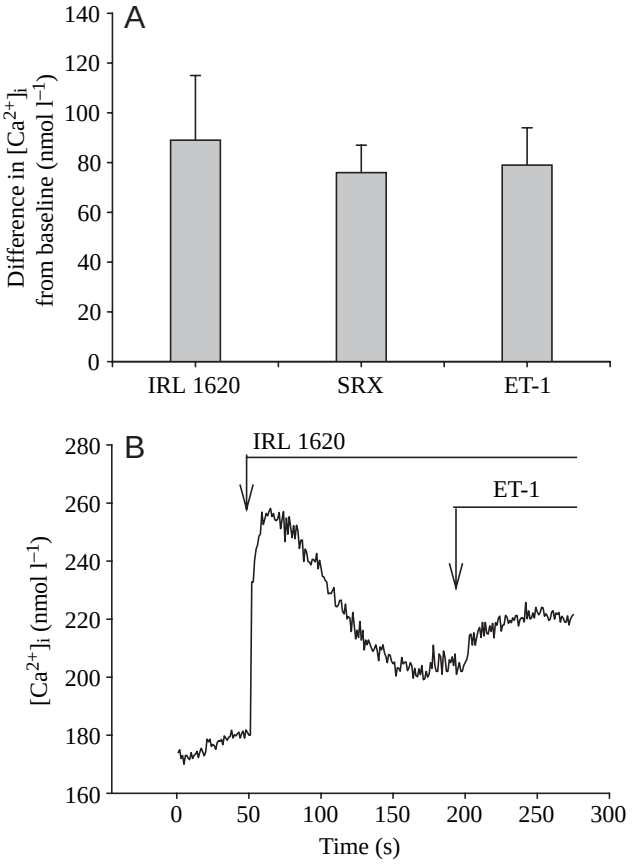

Fig. 1. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) responses of shark VSM to ET_BR receptor agonists and endothelin-1 (ET-1). (A) The ET_BR agonists IRL 1620 and sarafotoxin S6c (SRX) produce the same increase in $[Ca^{2+}]_i$ as ET-1. (B) Representative tracing of the cytosolic calcium responses of shark anterior mesenteric artery VSM. The ET_BR agonist IRL 1620 causes a prompt increase in $[Ca^{2+}]_i$. In the continued presence of IRL 1620, addition of ET-1 does not cause any further significant peak increase in $[Ca^{2+}]_i$.

increase in $[Ca^{2+}]_i$; the absolute value of the IRL 1620 peak was 366±22, the nadir was 270±31 and that of the subsequent ET-1 peak 282±30 nmol l^{-1} (N=8, P=0.78 for ET-1 *vs* IRL 1620 nadir). These results suggest but do not prove with certainty that ET_BR is the exclusive ET-1 receptor in *S. acanthias*.

Response to IRL-1620 and endothelin-1 in Ca^{2+}-Ringer

To confirm that the $[Ca^{2+}]_i$ responses to IRL 1620 and ET-1 were not different in Ringer containing Ca^{2+} (Ca^{2+}-Ringer) compared to Ca^{2+}-free Ringer, that is, both in the presence of Ca^{2+} mobilization and entry and without the possibility that SR depletion of Ca^{2+} was an issue, we performed experiments in Ca^{2+}-Ringer. The increase in $[Ca^{2+}]_i$ from baseline following IRL 1620 was149±12 nmol l^{-1} (N=7) and that following ET-1 was 146±20 nmol l^{-1} (N=5), demonstrating again that only ET_B receptor activity is present in shark VSM (data not shown).

Evidence for activation of the IP_3 receptor

Having established that stimulation of ET_BR but not ET_AR mobilized $[Ca^{2+}]_i$ from the SR, we investigated the mechanisms by which this occurs. To determine if ET_BR-coupled G protein activation results in formation of IP_3, activation of the IP_3R and mobilization of Ca^{2+} from the SR, we tested two inhibitors of the IP_3R in nominally Ca^{2+}-free Ringer. In the presence of TMB-8 (10^{-5} mol l^{-1}; Palade et al., 1989), IRL 1620 increased $[Ca^{2+}]_i$ by only 26.8±8 nmol l^{-1} (N=8, P<0.01 *vs* IRL without TMB-8). The compound 2-APB not only inhibits the IP_3R but also blocks Ca^{2+} entry *via* a store-operated Ca^{2+} entry channel in mammals (Broad et al., 2001; Maruyama et al., 1997) and in the dogfish shark (Fellner and Parker, 2002). In Ca^{2+}-free Ringer, only the effect on the IP_3R can occur. At concentrations of greater than 100 μmol l^{-1}, 2-APB can inhibit the SR/ER Ca^{2+}-ATPase (SERCA), leading to a rise of $[Ca^{2+}]_i$ (Peppiatt et al., 2003). In the presence of 2-APB (50 μmol l^{-1}) there was no change in baseline $[Ca^{2+}]_i$ (P=0.88) and the $[Ca^{2+}]_i$ response to IRL-1620 was reduced to 25.3±8 nmol l^{-1} (N=10, P<0.01 *vs* IRL without 2-APB). Thus TMB-8 and 2-APB inhibited the IRL 1620 response by 71% (Fig. 2A,B).

Evidence for activation of the ryanodine receptor by cADPR

Cyclic ADP-ribose is synthesized from NAD by the action of cADPR, which then stimulates the RyR to release Ca^{2+} from the endoplasmic/sarcoplasmic reticulum in sea urchin eggs and a variety of mammalian cell types (Guse, 2000; Lee, 2001). If the same systems operate in the shark, one might anticipate that ET_BR agonist stimulation of shark VSM would cause activation of the ADPR cyclase, formation of cADPR and opening of the RyR to release Ca^{2+} from the SR. To investigate this possibility, we treated VSM with 8-Br cADPR (1 μmol l^{-1}), a cell-permeant inhibitor of activation of the RyR. The $[Ca^{2+}]$ response to IRL 1620 was reduced to 53±8 nmol l^{-1} (N=16, P<0.01 for IRL alone *vs* IRL with 8-Br cADPR). 8-Br cADPR alone caused an increase in $[Ca^{2+}]_i$ of 16±6 nmol l^{-1}, which was not different from baseline values. Fig. 3A is a representative tracing of the response of shark VSM to IRL 1620 in the presence of 8-Br cADPR.

To further address the participation of the RyR in the response to activation of the ET_BR, we treated shark VSM in Ca^{2+}-free Ringer with lower concentrations of ryanodine (5 μmol l^{-1}) to activate rather than close the RyR. This concentration of ryanodine increased $[Ca^{2+}]_i$ by 49±8 nmol l^{-1} from baseline values (N=14, P<0.01). Subsequent addition of IRL 1620 further increased $[Ca^{2+}]_i$ by only 28±6 nmol l^{-1} (Fig. 3B,C). This value is significantly lower than that achieved by IRL 1620 following inhibition of the RyR with 100 μmol l^{-1} ryanodine, Ruthenium Red (*vide infra*) and 8-Br cADPR (P=0.02). Ryanodine at high concentrations (>>10 μmol l^{-1}) locks the RyR in a closed state (Carroll et al., 1991). Ryanodine (100 μmol l^{-1}) did not change baseline values of $[Ca^{2+}]_i$ (N=11, P=0.47). Subsequent addition of IRL 1620 increased $[Ca^{2+}]_i$ by 54.3±5 nmol l^{-1} (N=11, P=0.01 *vs* IRL 1620 without ryanodine; Fig. 3B). When shark anterior

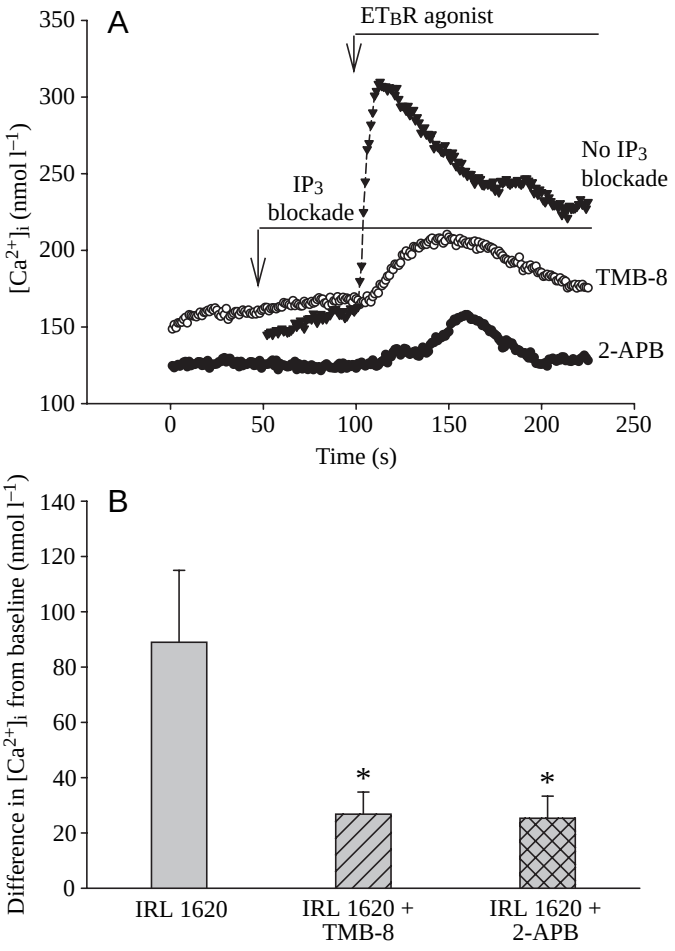

Fig. 2. Effect of the IP_3R blockers, TMB-8 and 2-APB, on the response of shark VSM to the ET_BR agonist IRL 1620 in Ca^{2+}-free Ringer. (A) Representative tracings of the response of VSM to ET_BR agonist stimulation in the presence or absence of TMB-8 or 2-APB. (B) Both inhibitors block the $[Ca^{2+}]_i$ mobilization response by about 70% (*P<0.01).

mesenteric artery VSM was pretreated with Ruthenium Red (50 μmol l^{-1}), an inhibitor of the RyR, basal $[Ca^{2+}]_i$ was unchanged and the addition of IRL 1620 caused an increase in $[Ca^{2+}]_i$ of 57±5 nmol l^{-1} (N=6, P<0.05 *vs* IRL 1620 without Ruthenium Red). Taken together, the three inhibitors of the RyR diminished the response of VSM to IRL 1620 by a mean of 39% (Fig. 3D).

Discussion

In mammals, the ET_AR is considered to be the predominant endothelin receptor subtype responsible for vasoconstriction in VSM. In rats, ET_BR agonists failed to induce vasoconstriction in pulmonary artery, carotid artery or renal artery (Evans et al., 1999; Marano et al., 1999; Seo and Luscher, 1995). However, in small mesenteric arteries, ET_BR accounted for about one half of the $[Ca^{2+}]_i$ response to ET-1 (Touyz et al., 1995) and in afferent arterioles, ET_BR were responsible for about one third of the ET-induced increase in $[Ca^{2+}]_i$ (Fellner and Arendshorst, 2004). Activation of both ET_AR and ET_BR appears to constrict renal afferent arterioles whereas primarily ET_BR stimulation constricted the efferent arteriole (Cavarape et al., 2003; Endlich et al., 1996). The dogfish shark appears to be unique in that ET-1 stimulation of vascular contraction in dorsal aorta and anterior mesenteric artery (Evans et al., 1996; Evans, 2001), or of increases in $[Ca^{2+}]_i$ in anterior mesenteric artery, occurred exclusively *via* ET_BR activation (this study). Evidence for ET_BR in teleost fish VSM was demonstrated in the bulbus arteriosus of the eel *Anguilla rostrata* in studies where there was dose-dependent constriction to ET-1 and SRX (Evans et al., 2003).

Having confirmed that ET_BR and not ET_AR seems to be the ET-1 subtype responsible for stimulating a rise in $[Ca^{2+}]_i$ in the anterior mesenteric artery of the dogfish VSM, we investigated the Ca^{2+} signaling pathways by which ET_BR activation elevates $[Ca^{2+}]_i$. To evaluate the participation of IP_3-mediated release of Ca^{2+} from the SR, we employed two inhibitors of the IP_3R, TMB-8 and 2-APB (50 μmol l^{-1}). In Ca^{2+}-free shark Ringer, these agents inhibited the response to ET_BR agonist stimulation by 71%. Although 2-APB has actions other than blockade of the IP_3R, in Ca^{2+}-free Ringer, store-operated Ca^{2+} entry cannot occur. While 2-APB can block SERCA Ca^{2+}-ATPase, it is reported to do so only at concentrations greater than 100 μmol l^{-1} (Peppiatt et al., 2003). TMB-8 has proven to be a reliable blocker of the IP_3R in VSM in the rat (Salomonsson and Arendshorst, 2001; Purdy and Arendshorst, 2001).

In mammalian VSM, the RyR participates in the process of Ca^{2+} mobilization following agonist stimulation of VSM. Stimulation of the RyR can occur *via* several separate mechanisms: calcium-induced calcium release (CICR) is a process by which an elevation of $[Ca^{2+}]_i$ (e.g. following activation of the IP_3R or entry through a voltage-gated Ca^{2+} channel) is thought to stimulate the RyR, thereby augmenting the signal (Galione and Churchill, 2002; Lee, 1993); alternatively, IP_3 may enhance ryanodine or cADPR binding to the RyR, creating a site of crosstalk between the two signaling pathways (Yusufi et al., 2002). Another mechanism involves the effect of cADPR on the RyR; cADPR-sensitive Ca^{2+} stores have been described in a broad variety of cell types in rat and mouse (Lee, 2001). In mammals, cADPR is both synthesized and hydrolyzed by the bifunctional protein CD38 (White et al., 2003). There are only two reports of cADPR activity in fish, both in oocytes (Fluck et al., 1999; Polzonetti et al., 2002). The current study is the first to demonstrate activity of cADPR in VSM of an elasmobranch, *S. acanthias*. Utilizing three different inhibitors of the RyR, including 8-Br cADPR, we found that the response to ET_BR agonist stimulation in Ca^{2+}-free shark Ringer was inhibited by 39%. These data are the first to demonstrate that the RyR participates in the elevation of $[Ca^{2+}]_i$ that is ultimately responsible for vascular contraction in the dogfish shark. Whether endothelin-1 directly activates the ADPR cyclase to form cADPR in the shark has not yet been studied in VSM of any species.

That the sum of inhibition achieved by inhibitors of IP_3R

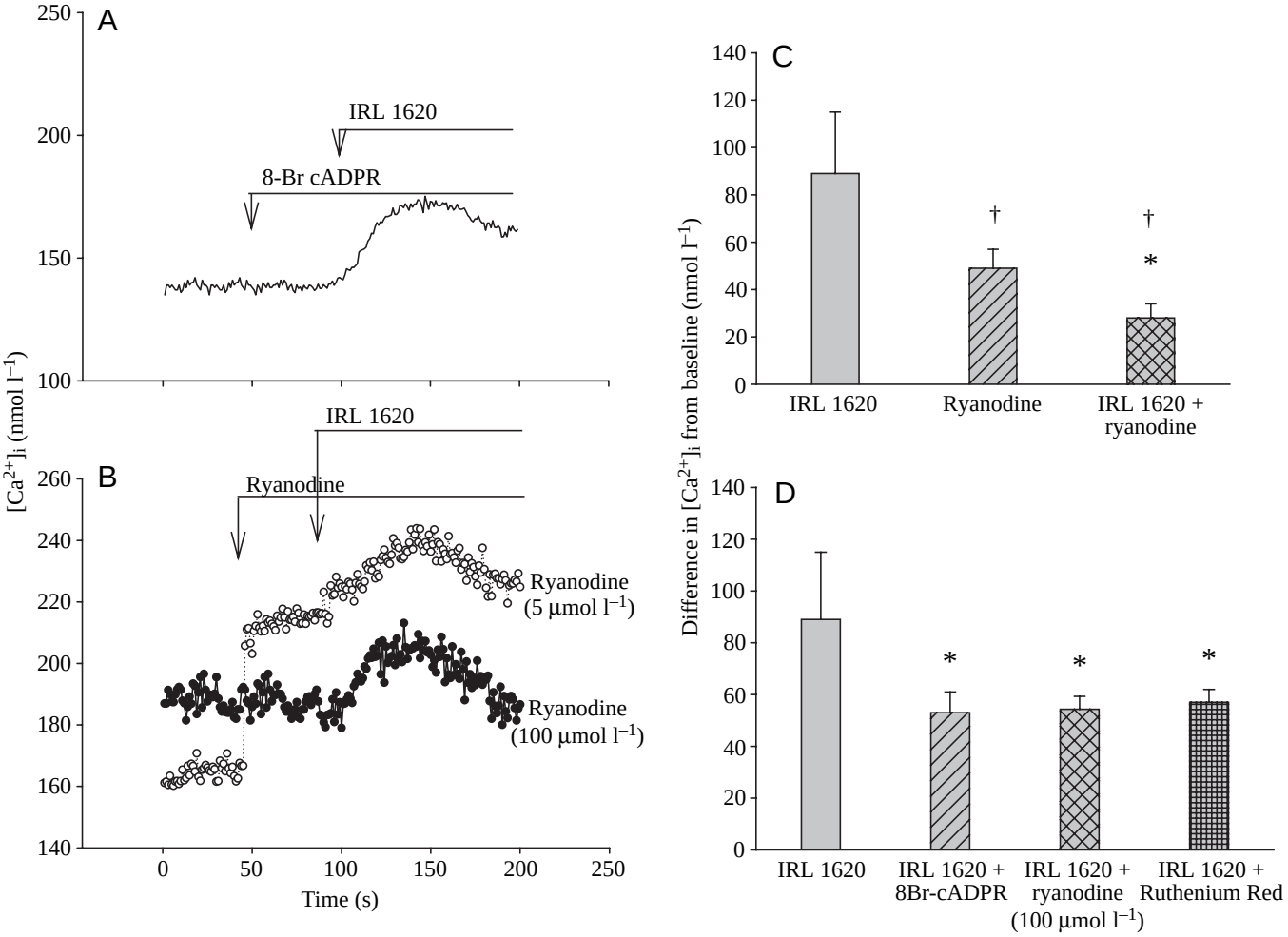

Fig. 3. Participation of the RyR in the $[Ca^{2+}]_i$ response to the ET_BR agonist IRL 1620 in shark mesenteric VSM. (A) Representative tracing of the diminished $[Ca^{2+}]_i$ response to IRL 1620 in the continued presence of the cADPR antagonist 8-Br cADPR. (B) Typical tracings of the stimulatory effect of a low concentration of ryanodine on the RyR and blocking effect of high concentrations of ryanodine. IRL 1620 was added in the continued presence of ryanodine. (C) Summary data of the response of VSM to a concentration of ryanodine that activates the RyR (5 μmol l^{-1}), followed by response to the ET_BR agonist IRL 1620 in the continued presence of ryanodine. The ryanodine and IRL 1620 plus ryanodine results were different from each other (*P<0.05) and both were different from IRL 1620 alone (P<0.05). (D) Three inhibitors of the RyR, 8Br-cADPR, high concentration of ryanodine (100 μmol l^{-1}) and Ruthenium Red, block the response of VSM to the ET_BR agonist IRL 1620 by approximately 39% (*P<0.01).

and RyR was greater than 100% raises the question of whether there is communication between the IP_3R- and RyR-responsive compartments within the SR. In canine pulmonary artery, for example, the SR of VSM appears to have two spatially distinct compartments, whereas in renal artery they communicate (Janiak et al., 2001), demonstrating that there is heterogeneity of calcium stores in vascular beds of a single species. Future studies will be required to examine the compartmental organization of Ca^{2+} stores in the shark SR.

The $[Ca^{2+}]_i$ response of shark anterior mesenteric artery VSM to ET_BR stimulation in Ca^{2+}-free Ringer was characterized by a prolonged peak of approximately 100 s. This contrasts with the short duration spike (<25 s) typical of the responses seen in mammalian VSM (Schroeder et al., 2000). The current studies were performed intentionally at 20–21°C, because shark cells are accustomed to ambient temperatures of 13–17°C in the sea, and our experience is that they lose viability at temperatures above 25°C. It has previously been shown that the removal of Ca^{2+} from the cytosol in mammalian cells is prolonged at reduced temperatures (13–27°C; Shuttleworth and Thompson, 1991), which may explain the long-duration peak response that we observed.

Previous studies of dogfish ventral aorta demonstrated that acetylcholine produced a concentration-dependent contraction of vascular rings whether or not the endothelium was intact (Evans and Gunderson, 1998). Furthermore, L-arginine, sodium nitroprusside and nitric oxide (NO) produced significant contractions, a finding opposite to that produced in mammalian VSM, in which dilation occurs with these

substances. More recently, it has been shown that there is evidence for a vasodilatory effect of NO in *S. acanthias in vivo*, in animals that were made severely hypoxic (E. Swenson, unpublished results). These results suggest that under ordinary conditions, endothelial cell ET_BR has little or no role in vascular homeostasis in *S. acanthias*.

One might ask if ET_BR is the only ET-1 receptor type in elasmobranchs. In shark rectal gland tubules, ET-1 regulates the xenobiotic efflux pump multidrug resistance-associated protein iosform 2 (MRP2). Inhibitors of ET_BR but not ET_AR prevented transport (Miller et al., 2002). The gill of the dogfish shark appears to express only ET_BR; the ET_BR-specific agonists IRL 1620, SRX and BQ-3020 competed against $^{(125)}$I-ET-1 at a single site (Evans and Gunderson, 1999). A band of muscle tissue has been identified on the periphery of the shark rectal gland. ET-1 constricted the smooth muscle but the ET_BR agonist SRX did not, suggesting that ET_AR might be the receptor involved (Evans and Piermarini, 2001). Inhibitors of ET_AR were not tried. It appears that ET_BR is an ancient receptor and that ET_AR, now generally considered to be the major vasoconstrictive sub-type of ET-1 in mammals, is a more recent evolutionary event.

In summary, we have established for the first time in VSM of the elasmobranch *Squalus acanthias* that stimulation of the ET_BR mobilizes Ca^{2+} from the SR *via* activation of both IP_3R and RyR and that cADPR participates in the signaling process. We have confirmed that ET_BR but not ET_AR may be the exclusive receptor for the endothelin in shark VSM.

List of abbreviations

cADPR	cyclic ADP-ribose
CICR	calcium-induced calcium release
ER	endoplasmic reticulum
ET	endothelin
ET-1	endothelin-1
ET_AR, ET_BR	endothelin A and B receptors
IP_3	inositol trisphophate
IP_3R	IP_3 receptor
MRP2	multidrug resistance-associated protein isoform 2
NAD	nicotinamide adenine dinucleotide
NO	nitric oxide
RyR	ryanodine-sensitive receptor
SERCA	SR/ER Ca^{2+}-ATPase
SR	sarcoplasmic reticulum
SRX	sarafotoxin S6c
TMAO	trimethylamine oxide
VSM	vascular smooth muscle

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