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Endothelin Receptor Type A Signals Both the Accumulation of Inositol Phosphates and the Inhibition of Cyclic AMP Generation in Rat Myometrium: Stimulation and Desensitization

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SUMMARY

In estradiol-dominated rat myometrium, endothelin (ET)-1 caused contraction and increased the accumulation of [^{3}H]inositol phosphates (EC_{50} = 70 nM), with the sequential generation of inositol trisphosphate, inositol bisphosphate, and inositol monophosphate. There was a coincident early decrease in phosphatidylinositol bisphosphate. The ET-1 stimulatory effect was pertussis toxin insensitive, suggesting an activation of phospholipase C via G_q/G_{11} proteins. ET-1 also inhibited the generation of cAMP induced by forskolin (EC_{50} = 30 nM). The inhibition was maintained in Ca^{2+}-depleted medium and was prevented by pertussis toxin, suggesting G_i-mediated inhibition of adenylyl cyclase. The rank order of potency for these various ET-1 effects [ET-1 > (Thr^2)-sarafotoxin-b ≫ ET-3], as well as the inhibitory effect displayed by BQ123, a specific ET_A receptor antagonist, provided evidence for the involvement of the ET_A receptor subtype. Exposure to ET-1 (15 min) resulted in concentration-dependent and homologous desensitization (40%) of the inositol phosphate response triggered by ET-1. There was virtually no recovery of ET-1-mediated inositol phosphate responses in the desensitized tissue even after 180 min of incubation. In contrast, the persistent low level of ET-1 activity that was observed in spite of several washings and in the absence of rechallenge with ET-1 was progressively reversed and totally eliminated by BQ123. The ET-1 inhibitory effect on cAMP was also desensitized, as evidenced by the attenuation of the inhibitory effect of ET-1 after 15 min of ET-1 pretreatment. The data indicate that in rat myometrium the ET_A receptor is coupled, via two distinct G proteins, to two main signal transduction cascades, which both undergo rapid desensitization.

ET, a 21-amino acid peptide originally isolated from porcine endothelial cells, belongs to a family of structurally homologous peptides that includes ET-1, ET-2, and ET-3, which are found in mammalian tissues, and sarafotoxins, which are constituents of the venom of *Actractaspis engaddensis* (1, 2). In addition to their vasoconstrictive activity, ETs have a multitude of biological effects that are mediated by multiple ET receptors with different specificities for the three peptides (3, 4). Pharmacological studies reveal that there are at least three distinct ET receptors. cDNAs for these receptors have been cloned, and functional characterization reveals that they are members of the G protein-coupled receptor superfamily (3, 5, 6). The first, designated the ET_A receptor, shows high affinity for ET-1, with the order of affinity for the ET isoforms being ET-1 = ET-2 ≫ ET-3; the second receptor, the ET_B receptor, exhibits equal affinities for the three ET isoforms, whereas the third receptor, the ET_C receptor, is selectively stimulated by ET-3 (6, 7). ET receptors have been demonstrated to modulate the production of multiple second messenger molecules, including $InsP_3$, diacylglycerol, arachidonic acid, and cAMP (2–4, 8).

Bousso-Mittler *et al.* (9) identified ET/sarafotoxin binding sites in intact rat uterus and demonstrated that the contractile activity of ETs was associated with an increase in inositol phosphate accumulation. However, the regulatory processes contributing to the generation of inositol phosphates and the nature of the phospholipid precursor were not fully characterized. The ability of ET-1 to contract isolated nonpregnant and late-pregnant rat uterus has also been documented recently (10). On the other hand, Maggi *et al.* (11, 12) reported that, for rabbit uterus, ET was produced by the endometrium, whereas specific ET receptors were located in the myometrium. A potential paracrine role for ET-1 was also proposed by Economos *et al.* (13), who demonstrated ET-1 gene expression and protein biosynthesis in human myometrium. These various findings suggested that the uterus might be a major site of ET action. The present study complements these preliminary observa-

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ABBREVIATIONS: ET, endothelin; SRTX-b, sarafotoxin-b; (Thr^2)-SRTX-b, (threonine^2)-sarafotoxin-b; $InsP_3$, inositol trisphosphate; $InsP_2$, inositol bisphosphate; InsP, inositol monophosphate; $PtdInsP_2$, phosphatidylinositol bisphosphate; PtdInsP, phosphatidylinositol monophosphate; PtdIns, phosphatidylinositol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IBMX, isobutylmethylxanthine.

tions. The data demonstrate that, in estrogen-treated rat myometrium, the potent contractile agonist ET-1 interacts with a specific ET_A receptor subtype, resulting in both activation of the phospholipase C system and inhibition of the adenylyl cyclase pathway, involving pertussis toxin-insensitive and -sensitive G proteins, respectively. Evidence is also presented for the rapid development of an agonist-specific refractoriness at the level of both inositol phosphate stimulatory and cAMP inhibitory effects triggered by ET-1.

Materials and Methods

Chemicals. *myo*-[2-^{3}H]Inositol (15–20 Ci/mmol) was from Amersham International (Amersham, Bucks, UK); ET-1, ET-3, and BQ123 were from Novabiochem (Cléryen Vexin, France). LiCl, oxytocin, carbamoylcholine chloride (carbachol), and β-estradiol 3-benzoate were obtained from Sigma Chemical Co. (St. Louis, MO), IBMX from Aldrich Chemical Co. (Milwaukee, WI), and forskolin from Calbiochem (Los Angeles, CA). cAMP was from P-L Biochemicals Inc. (Milwaukee, WI), [^{3}H]cAMP (38 Ci/mmol) from New England Nuclear Product Division (DuPont de Nemours, Paris, France), and pertussis toxin from List Biological Laboratories (Campbell, CA). Silica gel plates were from Merck (Darmstad, Germany). (Thr^2)-SRTX-b, a synthetic analog of SRTX-b possessing a threonine instead of a serine on position 2, was a generous gift of Dr. Lamthanh (CEA, Saclay, France). (Thr^2)-SRTX-b was shown to be as potent as SRTX-b in the contraction of rat uterus (14). Other chemicals were of the highest grade commercially available.

Animals and tissue processing. Immature Wistar female rats (4–5 weeks of age) were treated with 30 μg of estradiol for 2 days and were killed by decapitation the following day. Uteri were removed and the myometrium was prepared free of endometrium as described previously (15, 16).

Measurement of [^{3}H]inositol phosphates. Incubations were carried out as described (15, 16). Briefly, myometrial strips (about 40–50 mg, wet weight) were equilibrated at 37° for 20 min in 5 ml of Krebs bicarbonate buffer, pH 7.4, containing 117 mM NaCl, 4.7 mM KCl, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 0.8 mM $CaCl_2$, and 1 mM glucose (gas phase, O_2/CO_2, 19:1), under constant agitation. Tissues were then labeled with 5 μCi of *myo*-[2-^{3}H]inositol (0.4 μM) in 1 ml of fresh buffer for 4 hr, by which time the incorporation of [^{3}H]inositol into inositol lipids had reached a plateau (16). Myometrial strips were washed three times with 20 ml of nonradioactive Krebs buffer, transferred into 1 ml of fresh buffer, and incubated for 5 min before the addition of 10 mM LiCl. After 10 min, the agent to be tested was added at the indicated concentration and incubation was continued for the time indicated for the specific experiments. In the desensitization experiments, myometrial strips were initially exposed to the indicated concentration of ET-1 for 15 min. The tissue strips were then washed three times with 20 ml of peptide-free buffer, transferred to 1 ml of fresh buffer, and allowed to equilibrate for 2 min at 37°. Rechallenge experiments were routinely conducted in the presence of 10 mM LiCl and 0.2 μM ET-1 (or 0.1 μM oxytocin) for 13 min. In all experiments, the reactions were stopped by immersion of the tissue strips in 1.5 ml of cold 7% (w/v) trichloroacetic acid, followed by homogenization and centrifugation at 3000 × *g* for 15 min at 4°.

The trichloroacetic acid-soluble supernatants were extracted with 4 × 6 ml of diethyl ether, neutralized with Tris base, and applied to a column (0.7 × 2 cm) of the anion exchange resin (AG1-X8, formate form, 200–400 mesh) used for the separation of individual inositol phosphates, as described previously (15, 16). Alternatively, total inositol phosphates, i.e., $InsP_3$ plus $InsP_2$ plus InsP, were eluted together in a single step with 12 ml of 1 mM ammonium formate/0.1 M formic acid. The ^{3}H content of the various fractions was determined by scintillation counting in Quicksafe (Zinsser Analytic). Results were expressed as cpm/100 mg of tissue or, alternatively, as a percentage of stimulation over the basal values obtained before the addition of the stimulatory agonist.

Measurements of [^{3}H]phosphoinositides. The pellets obtained after centrifugation of the trichloroacetic acid homogenates were washed with 0.5 ml of trichloroacetic acid to remove any residual [^{3}H]inositol. Chlolroform/methanol/12 M HCl (40:80:1, v/v/v) (2.8 ml) was then added and the phospholipids were extracted for 30 min at room temperature (17). Chloroform (930 μl) and 0.1 N HCl (1700 μl) were then added and two phases were obtained by centrifugation. The upper phase was discarded, and the lower phase was dried under N_2. The dried lipid residues were dissolved in 100 μl of chloroform/methanol (95:5, v/v) and an aliquot of the extracts was spotted on silica gel thin layer plates that had been presoaked in 2 mM EDTA. PtdIns, PtdInsP, and $PtdInsP_2$ were separated by developing the plates in chloroform/methanol/4 M NH_4OH (90:70:20, v/v/v) (18). The phospholipids were located according to their migration, compared with authentic standards (detected with iodine vapor). The corresponding areas were cut out and placed into vials for determination of radioactivity by liquid scintillation counting.

Assay of cAMP levels. Myometrial strips (about 25 mg) were allowed to equilibrate at 37° for 20 min. The strips were then transferred into 1 ml of fresh buffer and allowed to equilibrate for 20 min. After a 3-min exposure to IBMX (final concentration, 240 μM), tissues were treated with 30 μM forskolin alone or combined with ETs at the indicated concentrations. Incubations were continued for 7 min. Reactions were stopped by immersion of the myometrial strips into 1.5 ml of cold 7% (w/v) trichloroacetic acid, followed by homogenization and centrifugation at 10,000 × *g* for 15 min at 4°. The trichloroacetic acid supernatants were extracted with diethyl ether, and cAMP was estimated according to the method of Gilman (19), as described previously (15). The centrifuged pellets were used for protein determination (20). cAMP was expressed as picomoles/milligram of protein.

Methods for recording uterine contractile responses. The contractile activity of isolated myometrial strips was measured with an isometric transducing device. The segments were loaded at a basal tension of 0.2–0.3 g and bathed at 37° in 10 ml of Krebs buffer, under 95% O_2/5% CO_2. The contractile activity was integrated during 1-min exposure to the agonist. Cumulative dose-response curves were obtained for each agonist. Responses were calculated as a percentage of the maximum response induced by ET-1 and were plotted against the logarithm of the agonist concentration. When the antagonist BQ123 was used, contractions were induced initially with ET-1 and then increasing concentrations of BQ123 were added at 1-min intervals.

Data analysis. The results are expressed as means ± standard errors and were analyzed statistically using Student's *t* test. A *p* value of <0.05 was considered significant.

Results

Stimulatory effects of ETs on uterine contraction. Data in Fig. 1 illustrate the dose-dependent efficacy of ET-1 to elicit contractions in rat myometrium (EC_{50} of 0.7 ± 0.053 nM and maximal effect at 10 ± 0.55 nM). It was interesting to note that the maximal contractile activity induced by ET-1 was similar to that induced by the strong uterine contractile agonist oxytocin (data not shown). ET-3 also elicited contractions but appeared less potent, with a concentration-dependent curve that lay to the right of the dose-response curve for ET-1. An EC_{50} of 60 ± 7.6 nM was estimated for ET-3, with a maximal contractile response being reached at 200 nM. This order of potency, ET-1 > ET-3, suggested that an ET_A receptor subtype was associated with ET-mediated uterine contractions, which appeared in close agreement with recently reported observations (9, 10, 21). Our interpretation was further supported by the loss of ET-1 effects on contraction in the presence of BQ123, a specific ET_A receptor antagonist (22, 23) (Fig. 1). BQ123 failed to affect contractions of similar magnitude induced by carbachol (data not shown).

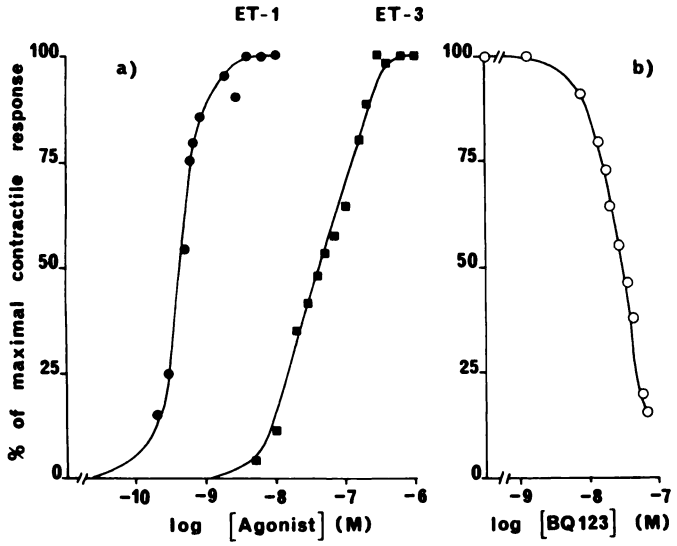

Fig. 1. Contractile response of myometrium to various concentrations of ET-1 and ET-3 and effect of BQ123. Isometric contractions were recorded during 1-min exposure of loaded myometrial strips to the indicated concentrations of ET-1 (●) and ET-3 (■) (a) and to the indicated concentrations of BQ123 after stimulation of myometrium with 3.5 nM ET-1 (b). Values are means of four independent experiments and agreed within 10%.

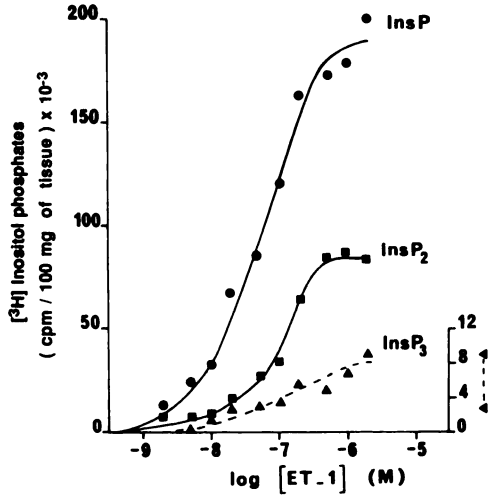

Fig. 2. Dose-dependent effects of ET-1 on the accumulation of inositol phosphates in rat myometrium. [^{3}H]Inositol-prelabeled myometrial strips were incubated for 10 min in the presence of 10 mM LiCl and then stimulated for an additional 13 min with the indicated concentrations of ET-1. Incubations were stopped with 7% trichloroacetic acid and the individual [^{3}H]inositol phosphates, i.e., $InsP_3$ (▲), $InsP_2$ (■), and InsP (●), were separated on AG1-X8 columns as described in Materials and Methods. Values are means of three different experiments, each assayed in duplicate. The standard error did not exceed 10% of the mean.

Stimulatory effects of ET on $PtdInsP_2$ breakdown in rat myometrium. Data in Fig. 2 illustrate the stimulatory effects of ET-1 on the increased generation of inositol phosphates (InsP, $InsP_2$, and $InsP_3$) in myometrial strips incubated in the presence of 10 mM LiCl. The stimulatory effect of ET-1 was dose dependent. The sensitivity to ET-1 concentrations was the same for the generation of the three inositol phosphates, with EC_{50} values of 80 ± 9.8 nM, 80 ± 7.4 nM, and 70 ± 8.7 nM for $InsP_3$, $InsP_2$, and InsP, respectively, and maximal activation for the three inositol phosphates was similarly achieved at 0.2 μM ET-1. Maximal levels of accumulated $InsP_3$, $InsP_2$, and InsP were 4-, 25-, and 8-fold, respectively, over basal values, which amounted to 1,183 ± 193, 3,068 ± 263, and 18,453 ± 1,524 cpm/100 mg of tissue for $InsP_3$, $InsP_2$, and InsP, respectively. Data in Fig. 3 illustrate the comparative effects of ET-1, (Thr^2)-SRTX-b, and ET-3 to cause increased generation of total insitol phosphates (InsP plus $InsP_2$ plus $InsP_3$). Compared with ET-1, which displayed an EC_{50} of 70 ± 5.7 nM and a maximal reponse (1100% stimulation over basal levels) achieved at 0.2 μM, (Thr^2)-SRTX-b appeared slightly less potent (EC_{50} = 100 ± 8.5 nM) and displayed a smaller effect (70% of ET-1 maximal response) at a maximal concentration of 100 μM. In marked contrast, there was a strong rightward shift in the concentration-response curve obtained with ET-3, with a stimulatory effect at 100 μM that did not exceed 40% of the response caused by ET-1. The rank order of potency [ET-1 > (Thr^2)-SRTX-b ≫ ET-3] was consistent with an ET_A receptor coupled to phospholipase C activation in the myometrium. This interpretation was further confirmed by the ability of BQ123, a selective ET_A receptor antagonist, to affect the ET-1-mediated inositol phosphate response. As shown in Fig. 4, exposure of the myometrium to BQ123 resulted in a dose-dependent inhibition of inositol phosphate accumulation induced by the subsequent addition of ET-1. Maximal inhibition was achieved at 5 μM BQ123, with a calculated K_i value (according to the Cheng and Prusoff relationship) of 70 ± 7.6 nM. The specificity of the interaction of BQ123 with ET receptors was evidenced by the data illustrated in Fig. 4, *right*, which clearly demonstrated the inability of BQ123 to block either carbachol- or oxytocin-mediated accumulation of inositol phosphates under conditions (1 μM) where the antagonist totally abolished the formation of inositol phosphates triggered by ET-1.

Data in Fig. 5 show the time course of changes in the radioactivity associated with the individual inositol phosphates after ET-1 stimulation of [^{3}H]inositol-prelabeled myometrial strips in the presence of LiCl. Upon addition of ET-1 at 200

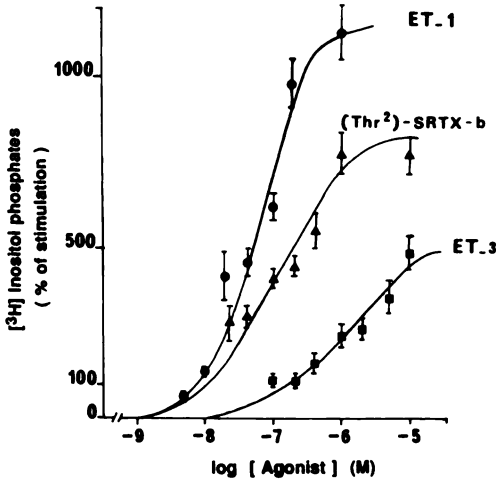

Fig. 3. Comparison of the dose-dependent effects of ET-1, ET-3, and (Thr^2)-SRTX-b on inositol phosphate accumulation. [^{3}H]Inositol-prelabeled myometrial strips were incubated with 10 mM LiCl for 10 min and then treated with the indicated concentrations of ET-1 (●), (Thr^2)-SRTX-b (▲), or ET-3 (■) for 13 min. Total inositol phosphates were determined as described in Materials and Methods. Results are expressed as percentage of stimulation over control value (21,218 ± 1,793 cpm/100 mg of tissue). Values are means ± standard errors of four or five experiments, each done in duplicate.

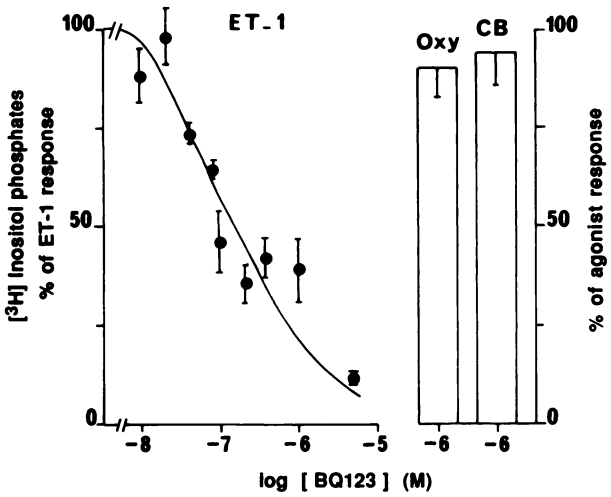

Fig. 4. Effect of the selective ET_A antagonist BQ123 on the accumulation of inositol phosphates induced by ET-1 and comparison with carbachol and oxytocin. [^{3}H]Inositol-prelabeled myometrial strips were treated with the indicated concentrations of BQ123 for 5 min. Tissues were then exposed to 10 mM LiCl for 10 min, followed by a 13-min incubation with 80 nM ET-1 (left panel), 50 μM carbachol (*CB*), or 100 nM oxytocin (*Oxy*) (right panel). Total inositol phosphates were determined as described in Materials and Methods. Results are expressed as percentage of the total inositol phosphate response to ET-1, carbachol, or oxytocin (198,243 ± 21,200, 149,311 ± 14,355, and 156,503 ± 14,720 cpm/100 mg of tissue, respectively). Values are means ± standard errors of three different experiments, each assayed in duplicate.

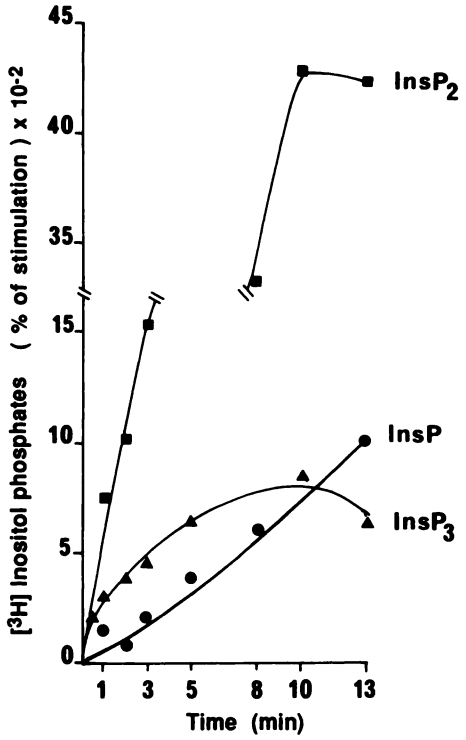

Fig. 5. Time course of ET-1-induced accumulation of inositol phosphates. [^{3}H]Inositol-prelabeled myometrial strips were incubated for 10 min in the presence of 10 mM LiCl before addition of 0.2 μM ET-1. Incubations were stopped with 7% trichloroacetic acid at the times indicated and the individual [^{3}H]inositol phosphates, i.e., $InsP_3$ (▲), $InsP_2$ (■), and InsP (●), were separated as described in Materials and Methods. Results are expressed as percentage of stimulation over control values (1,183 ± 193, 3,068 ± 263, and 18,453 ± 1,524 cpm/100 mg of tissue for $InsP_3$, $InsP_2$, and InsP, respectively). Values are means of seven independent experiments, each carried out in duplicate. The standard error did not exceed 10% of the mean.

nM, there was a rapid increase in $InsP_3$ and $InsP_2$ (120% and 100% stimulation, respectively, at 30 sec). The accumulation of InsP was delayed (100% stimulation at 2 min). $InsP_3$ and $InsP_2$ reached plateaus at 7 and 10 min, respectively, whereas InsP continued to accumulate and was not stabilized at 15 min, illustrating a sequential precursor-product relationship in the order of $InsP_3$, $InsP_2$, and InsP. Data in Fig. 6 show the distribution of [^{3}H]inositol in PtdIns, PtdInsP, and $PtdInsP_2$. In control tissue incubated in the presence of LiCl, most of the [^{3}H]inositol was incorporated into PtdIns (92% of the inositol phospholipid pool), whereas PtdInsP and $PtdInsP_2$ accounted for 3.4% and 4.6%, respectively. After the addition of ET-1 to myometrial strips, the $PtdInsP_2$ level decreased rapidly below the initial level, to reach values of 73% and 66% of control within 30 sec and 1 min, repectively. The amount of PtdInsP also decreased, albeit less rapidly, reaching values of 80% and 70% of control within 30 sec and 1 min, respectively. In contrast, the level of PtdIns was not altered significantly in the presence of ET-1 and remained constant for incubation times as long as 15 min. At that time, the levels of $PtdInsP_2$ and PtdInsP had almost totally recovered, indicating that these compounds were continuously replenished from the labeled PtdIns pool. The rapid decrease in the level of $PtdInsP_2$ at a time corresponding to the earliest rise in $InsP_3$ formation provided additional evidence that $PtInsP_2$ is the target for phospholipase C activation due to ET-1.

Fig. 7 further demonstrates the reversibility of the ET-1 receptor-dependent changes in the accumulation of the three inositol phosphates. When BQ123 was added after 13 min of stimulation with ET-1 in the presence of LiCl, there was a progressive return to the unstimulated values for both $InsP_3$ and $InsP_2$. However, the earliest change was seen for $InsP_3$, which immediately began to decrease ($t_{1/2}$ = 2.8 min), reaching basal values at 10 min, whereas the decrease in $InsP_2$ was delayed ($t_{1/2}$ = 8 min). The situation was different with InsP, which continued to accumulate at the same rate for 4–5 min after addition of BQ123; its generation then stopped totally. However, as opposed to $InsP_3$ and $InsP_2$, the decrease in InsP was hardly detectable even 15 min after the addition of the ET_A receptor antagonist. These data provide strong evidence that the generation of the three inositol phosphates is totally agonist dependent, i.e., it reflects the continuous interaction of ligand-free receptors with ET-1. Finally, the time sequence of the disappearance of inositol phosphates upon removal of the stimulatory agonist, in the order of $InsP_3$, $InsP_2$, and InsP, closely resembled their time sequence of generation at the onset of ET-1 addition, supporting the interpretation that the primary substrate for ET_A receptor-mediated activation of phospholipase C is most probably $PtdInsP_2$. Another noteworthy observation revealed in Fig. 7 concerns the time-dependent decrease in the level of $InsP_3$ and $InsP_2$ detected even in the absence of any added antagonist. The phenomenon is discussed below in terms of ET receptor desensitization.

Effect of pertussis toxin on ET-1-induced accumulation of inositol phosphates. In previous experiments (16), it was found that treatment of myometrial strips with fluoroaluminates caused a marked increase in inositol phosphate generation, providing indirect evidence for the involvement of a G protein in the activation of phospholipase C. We further dem-

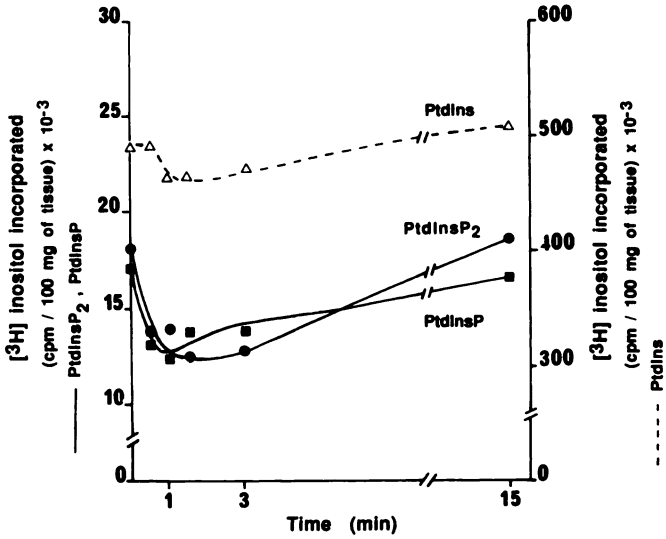

Fig. 6. Changes in the [^{3}H]inositol incorporation into phosphoinositides after stimulation of myometrium with ET-1. After a 10-min incubation in the presence of 10 mM LiCl, [^{3}H]inositol-prelabeled myometrial strips were incubated with 0.2 μM ET-1. At the indicated times, the incubations were stopped with 7% trichloroacetic acid and the ^{3}H-labeled phospholipids were extracted and separated by thin layer chromatography on silica gel plates, with a solvent system of $CHCl_3/CH_3OH/4$ mM NH_4OH (90:70:20, v/v/v). The lipids were located according to their migration compared with authentic standards (detected with iodine vapor), cut out, and counted for radioactivity as described in Materials and Methods. Incorporation of ^{3}H into PtdIns (△), PtdInsP (■), and $PtdInsP_2$ (●) is expressed as cpm/100 mg of tissue. Values are means of three different experiments, each done in duplicate. The standard error did not exceed 10% of the mean.

onstrated that incubation of myometrial strips with a dose of 300 ng/ml pertussis toxin for 6 hr, conditions under which the pertussis toxin-sensitive G proteins in the cells are completely ADP-ribosylated (15, 24), failed to affect the production of inositol phosphates caused by a 10-min stimulation with 0.5 μM ET-1. The values for total [^{3}H]inositol phosphates in control and ET-1-stimulated myometrium were 22,704 ± 2,410 and 245,565 ± 23,000 cpm/100 mg of tissue for the untreated myometrial preparations and 21,286 ± 2,050 and 256,360 ± 24,000 cpm/100 mg of tissue for the pertussis toxin-treated preparations, respectively. Thus, in rat myometrium, the coupling of the activated ET_A receptor to phospholipase C is triggered by a pertussis toxin-insensitive G protein.

Desensitization of the inositol phosphate response to ET-1. As illustrated in Fig. 7, a gradual decrease in the rate of accumulation of the three inositol phosphates was observed during prolonged incubation with ET-1 even in the presence of LiCl. It was observed that the accumulation of total [^{3}H]inositol phosphates, used as a measure of phospholipase C activity, was linear for up to 13 min of incubation with ET-1. The decreased rate was detected first for $InsP_3$ and then for its metabolites, i.e., $InsP_2$ and InsP, indicating that the decreased accumulation of inositol phosphates observed after 13 min of ET-1 treatment was initiated by the reduced production of $InsP_3$.

The results of Fig. 6 clearly indicated that the attenuated inositol phosphate accumulation in response to ET-1 was not brought about by a limiting supply of the phospholipace C substrate. Indeed, after 15 min of treatment with ET-1, i.e., the time corresponding to a marked decline in the stimulatory effect of ET-1, the levels of $PtdInsP_2$ and PtdInsP, which had displayed a transient fall during the first 2 min of stimulation, had totally recovered. It was further verified (Table 1) that the decline in inositol phosphate responses was not due to any significant degradation of ET-1 or to the accumulation in the medium of an inhibitory product, because addition of the 30-min incubate to fresh tissue induced a corresponding normal increment in inositol phosphate generation. On the other hand, when myometrial strips that had been treated with ET-1 for 15 min were washed to remove the agonist and subsequently challenged with fresh ET-1 in the presence of LiCl, there was a diminished response (40%) in terms of inositol phosphate accumulation, compared with similarly treated tissue in ET-1-free medium. The marked fall in the inositol phosphate response most probably reflected the phenomenon of ET-1-induced refractoriness. Additionally, the data show that the trigger for desensitization is an early event.

As indicated in Fig. 8, the desensitization process was concentration dependent, with an EC_{50} of 50 ± 7.3 nM and a V_{max} of 0.2 μM for ET-1. These concentrations were similar to those required for the stimulatory process, indicating that ET-1-induced desensitization was an agonist receptor-mediated process. It is worth noting that, in the absence of any rechallenge with ET-1, addition of LiCl alone to desensitized myometrium

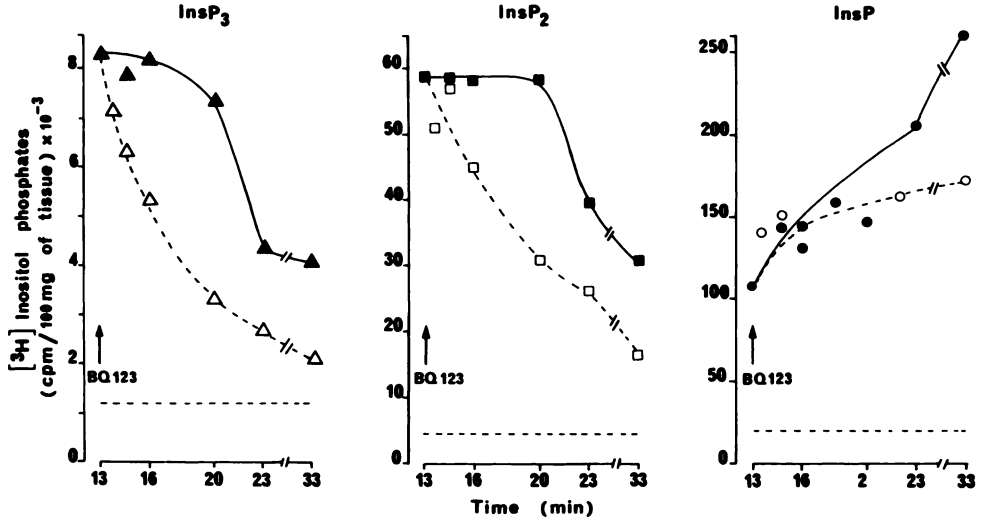

Fig. 7. Reversal by BQ123 of the stimulatory effect of ET-1 on inositol phosphate accumulation. [^{3}H]Inositol-prelabeled myometrial strips were incubated with 10 mM LiCl for 10 min before the addition of 0.2 μM ET-1. After a 13-min stimulation with ET-1, incubations were continued without (▲, ■, ●) or with (△, □, ○) the addition (*arrows*) of 1 μM BQ123. Incubations were stopped at the times indicated and the individual [^{3}H]inositol phosphates were separated as described in Materials and Methods. The changes in the accumulation of $InsP_3$ (▲, △), $InsP_2$ (■, □), and InsP (●, ○) were expressed as cpm/100 mg of tissue. Values are means of three independent experiments, each done in duplicate. The standard error did not exceed 10% of the mean.

TABLE 1

ET-1-induced desensitization of the inositol phosphate response

A, Control represents [^{3}H]inositol-prelabeled myometrial strips incubated for 13 min in the presence of LiCl (10 mM), with or without 0.2 μM ET-1. B, After exposure of [^{3}H]inositol-prelabeled myometrial strips to 0.2 μM ET-1 for 15 min, tissues were washed with 3 × 10 ml of peptide-free buffer, transferred into 1 ml of fresh buffer, and allowed to equilibrate for 2 min. LiCl (10 mM) was then added and the tissues were incubated for an additional 2 min before being rechallenged for 13 min with 0.2 μM ET-1. C, Myometrial strips were incubated for 13 min in the presence of 0.2 μM ET-1 and then removed, and the incubated medium was added to fresh myometrial strips. Incubations were carried out for an additional 13 min in the presence of 10 mM LiCl. The individual inositol phosphates were estimated as described in Materials and Methods. Values are means ± standard errors of three to seven experiments, each done in duplicate.

Additions	[^{3}H]inositol phosphates			
	Total	InsP	$InsP_2$	$InsP_3$
	cpm/mg of tissue			
A. Control				
LiCl	14,860 ± 1,681	11,340 ± 1,247	2,290 ± 274	1,230 ± 160
LiCl + 0.2 μM ET-1	267,850 ± 30,521	179,800 ± 19,778	79,160 ± 9,499	8,890 ± 1,244
B. 0.2 μM ET-1-treated tissue, washed and rechallenged				
LiCl	117,600 ± 10,171	98,070 ± 8,335	15,890 ± 1,509	3,640 ± 327
LiCl + 0.2 μM ET-1	159,420 ± 16,758	128,400 ± 13,482	27,170 ± 2,853	3,850 ± 423
C. Medium containing preincubated ET-1 (0.2 μM) + fresh tissue	288,120 ± 31,471	170,190 ± 17,870	110,055 ± 12,656	7,875 ± 945

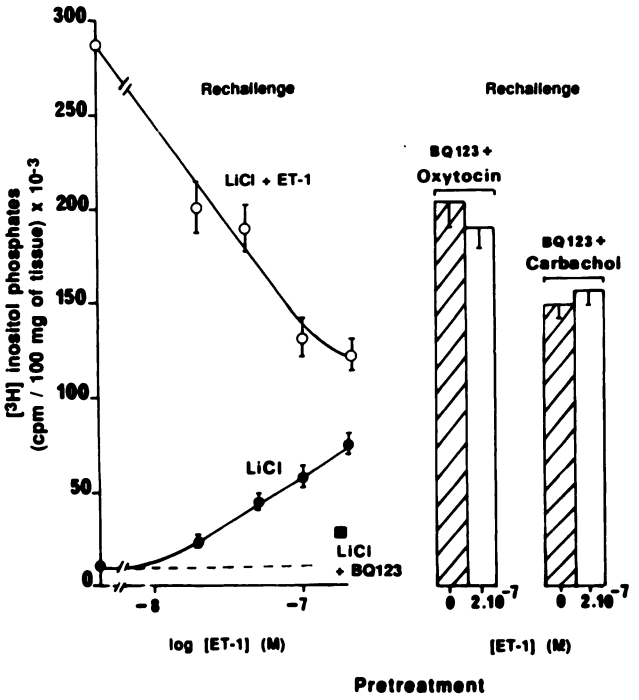

Fig. 8. Effect of various concentrations of ET-1 on the desensitization of the inositol phosphate response. [^{3}H]Inositol-prelabeled myometrial strips were initially exposed to the indicated concentrations of ET-1 for 15 min, washed with 5 × 10 ml of ET-1-free buffer, and allowed to equilibrate for 2 min in 1 ml of fresh buffer. Tissues were then rechallenged in the presence of 10 mM LiCl without (●, ■) or with (○) the addition, 2 min later, of 0.2 μM ET-1 for an additional 13 min (left panel). Where indicated (■), BQ123 at 1 μM was added 5 min before the addition of LiCl. For comparison with oxytocin and carbachol (right panel), [^{3}H]inositol-prelabeled myometrial strips were incubated for 15 min in the absence (■) or in the presence (□) of 0.2 μM ET-1, washed several times, transferred to fresh medium, and stimulated for 13 min with 0.1 μM oxytocin or 15 μM carbachol in the presence of 1 μM BQ123. Total inositol phosphates were determined as described in Materials and Methods. Values are means ± standard errors of at least four different experiments, each assayed in duplicate.

resulted in inositol phosphate accumulation larger than that in nondesensitized tissue. The extent of inositol phosphate production increased with increasing concentrations of ET-1 used during the initial incubation period, suggesting that, in spite of several washings, there was still some ET-1 bound to the myometrial preparations. This interpretation was confirmed by the observation that when BQ123, used at a maximally effective concentration, was added 15 min before LiCl the basal inositol phosphate response of tissue that had been maximally desensitized by ET-1 was similar to that of nondesensitized tissue.

We next determined the type of desensitization induced by a 15-min exposure to ET-1. Myometrial preparations that had been pretreated with the peptide were washed, treated with BQ123 to block the effect of residually bound ET-1, and rechallenged with two other $PtdInsP_2$-degrading agonists, i.e., carbachol and oxytocin. Fig. 8 shows that, under these conditions, neither carbachol nor oxytocin stimulatory effects were affected by the ET-1 pretreatment. A short exposure to ET-1, i.e., conditions where phospholipase C activity was not significantly affected, similarly failed to attenuate the subsequent response to oxytocin. The values for total [^{3}H]inositol phosphates triggered by oxytocin in the controls and in myometrial preparations after 5-min pretreatment with ET-1 averaged 205,000 ± 20,000 and 198,000 ± 21,000 cpm/100 mg of tissue, respectively. Thus, during the first 15 min, desensitization of ET-1-evoked inositol phosphate generation was an homologous process.

The next experiments were designed to evaluate the reversibility of the agonist-induced refractoriness. Myometrial strips were treated for 10 min with 0.2 μM ET-1, after which the tissues were washed and then further incubated in peptide-free medium for various periods of time. Inositol phosphate responses were assayed after a 13-min rechallenge with either LiCl alone (basal) or LiCl in the presence of ET-1. Data in Fig. 9 indicate that after a 30-min incubation in ET-1-free medium the levels of inositol phosphates in desensitized myometrium, attained in the presence of LiCl alone, were identical to the levels obtained in nondesensitized tissue, indicating that by that time ET-1 that had bound to the receptors and had not been removed by the washing had dissociated. Most dramatically, there was virtually no recovery of ET-1-mediated inositol phosphate responses in the desensitized tissue, even after 180 min of incubation in the absence of the peptide and rechallenge with fresh ET-1. Such a poorly recovered response of target

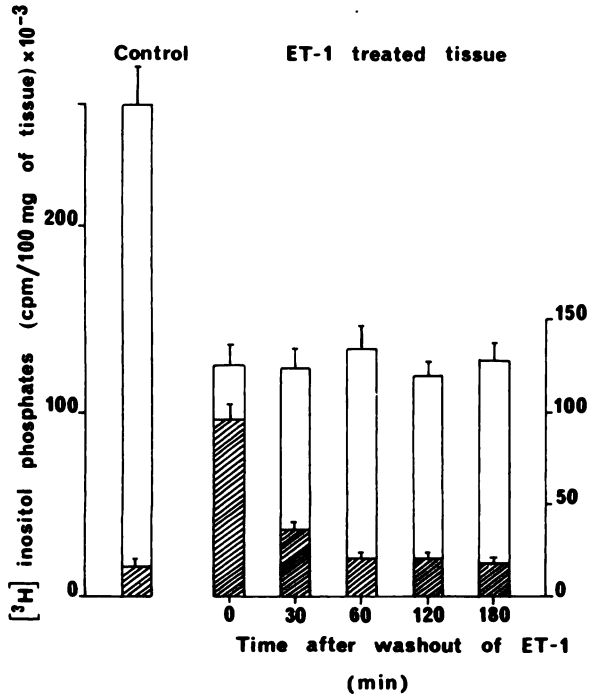

Fig. 9. Irreversibility of the ET-1-induced refractoriness in terms of [^{3}H] inositol phosphate responses. [^{3}H]Inositol-prelabeled myometrial strips were initially incubated in the absence (control) or in the presence of 0.2 μM ET-1 for 15 min (ET-1-treated tissue). The tissues were washed with 5 × 10 ml of peptide-free buffer and then incubated in 1 ml of fresh buffer. At the indicated times, tissues were rechallenged with 10 mM LiCl in the presence (□) or in the absence (■) of 0.2 μM ET-1. After a 13-min incubation, total inositol phosphates were estimated as described in Materials and Methods. The response of ET-1-treated tissue to a second addition of ET-1 was decreased by 45%, compared with the control. Values are means ± standard errors of four different experiments, each assayed in duplicate.

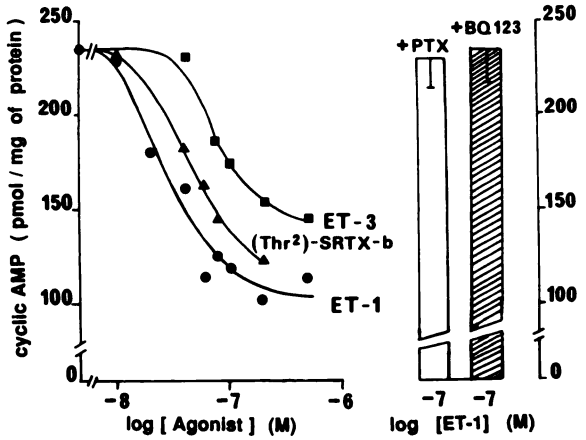

Fig. 10. Inhibitory effects of various concentrations of ET-1, (Thr^2)-SRTX-b, and ET-3 on forskolin-induced cAMP accumulation and effects of BQ123 and pertussis toxin. After a 5-min exposure to 150 μM IBMX, myometrial strips were treated for 1 min with the indicated concentrations of ET-1 (●), (Thr^2)-SRTX-b (▲), or ET-3 (■). Forskolin (30 μM) was then added and incubations were carried out for an additional 7 min (left panel). When used (right panel), BQ123 was added 5 min before exposure to 0.1 μM ET-1 and 30 μM forskolin (■). For pertussis toxin (*PTX*) treatment (□), myometrial strips were incubated for 6 hr in the presence of 300 ng/ml pertussis toxin, washed with fresh buffer, and then treated with 0.1 μM ET-1 and 30 μM forskolin. cAMP was extracted and assayed as described in Materials and Methods. Values are the means of three to five independent experiments, each done in duplicate. The standard error did not exceed 10% of the mean.

cells to ET-1, after desensitization triggered by the peptide, has been reported previously (25).

Inhibitory effect of ET on cAMP accumulation. Forskolin at 30 μM stimulated cAMP accumulation in rat myometrium from 4.5 ± 0.28 to 220 ± 14.2 pmol of cAMP/mg of protein, in the presence of the cAMP phosphodiesterase inhibitor IBMX. Addition of ET-1 dose-dependently reduced the cAMP accumulation triggered by forskolin (Fig. 10). (Thr^2)-SRTX-b and ET-3 also inhibited cAMP accumulation induced by forskolin. However, there were marked differences in the efficacy and the potency of inhibition induced by the three ET agonists. ET-1 was the most potent, with an EC_{50} of 30 ± 4.2 nM, followed closely by (Thr^2)-SRTX-b, with an EC_{50} of 40 ± 6.3 nM, whereas the EC_{50} for ET-3 was 10 times higher, i.e., 700 ± 72.5 nM. At their maximally effective concentrations [0.1 μM, 0.2 μM, and 1 μM for ET-1, (Thr^2)-SRTX-b, and ET-3, respectively], the extent of inhibition induced by ET-1 and (Thr^2)-SRTX-b averaged 45%, compared with 30% inhibition observed with ET-3. These results suggested that the type of ET receptor involved in the inhibition of cAMP accumulation was the ET_A receptor, i.e., the same type of receptor involved in the degradation of $PtdInsP_2$ and in the contractile activity. This result was further confirmed by the loss of the ET-1 inhibitory effect on cAMP levels in the presence of BQ123, the selective ET_A receptor antagonist.

Previous studies (26, 27), including our own,[1] demonstrated that stimulatory agonists were able to trigger a transient contractile activity when applied to myometrial strips incubated in a Ca^{2+}-depleted medium containing 1 mM EGTA. This was followed by a progressive decline in the ability of agonists to induce uterine contraction, with virtually no contraction being expressed after a 10-min exposure of the tissue to a Ca^{2+}-free medium, indicating that removal of extracellular Ca^{2+} could ultimately limit the ability of the sarcoplasmic reticulum to maintain its Ca^{2+} content (28). It was observed (Fig. 10) that the ET-1 inhibitory effect on forskolin-induced cAMP accumulation was maintained under such conditions of Ca^{2+} depletion, with neither basal nor forskolin-induced cAMP accumulation being modified (data not shown). Inhibition of forskolin-mediated cAMP accumulation caused by ET-1 averaged 45% in normal Ca^{2+}-containing medium and 44% in Ca^{2+}-depleted medium. Thus, the inhibitory effect of ET-1 did not result from a secondary effect mediated by an increase in intracellular Ca^{2+} levels subsequent to the degradation of $PtdInsP_2$ induced by ET-1. This interpretation was further substantiated by the data obtained with pertussis toxin (Fig. 10). The toxin, which had no effect on basal or forskolin-induced cAMP accumulation (15, 24), completely abolished the inhibitory effect of ET-1 on forskolin-induced cAMP accumulation. It was concluded that the inhibitory effect of ET-1 on cAMP accumulation was due to G_i-mediated inhibition of the cAMP-forming system.

Finally, when myometrium was exposed to ET-1 for 15 min, washed, and rechallenged with the peptide, there was a substantial decrement in the capacity of ET-1 to inhibit cAMP accumulation induced by forskolin (5% inhibition, compared with 45% for the nondesensitized tissue). Thus, under condi-

[1] Tanfin, Z., and S. Harbon, unpublished observations.

tions that resulted in the desensitization of the $PtdInsP_2$ responses to ET-1, there was also a desensitization of the inhibitory cAMP responses to the peptide.

Discussion

The data presented herein demonstrate that, in estrogen-treated rat myometrium, the potent contractile agonist ET-1 interacts with a specific ET_A receptor subtype, resulting in both activation of the phospholipase C transducing system via a pertussis toxin-insensitive G protein and inhibition of the adenylyl cyclase pathway via a pertussis toxin-sensitive G_i protein.

Analysis of the rate of accumulation of the three inositol phosphates indicates that $PtdInsP_2$ is the substrate for phospholipase C. Thus, upon addition of ET-1 to myometrium, $InsP_3$ was the first inositol phosphate accumulated, followed by $InsP_2$ and InsP. Under these conditions, the time point corrresponding to the early rise in $InsP_3$ was associated with a fall in $PtdInsP_2$. Also, when BQ123, a specific ET_A receptor antagonist, was added to myometrium that had been prestimulated with ET-1, there was a gradual decrease in the accumulation of the three inositol phosphates, with the earliest decrease being detected for $InsP_3$. The inhibitory effect of ET-1 on cAMP accumulation was evidenced by a reduction in the level of cAMP induced by forskolin. Maximum ET-1 inhibitory effect was achieved at 300 nM, with an EC_{50} of 30 ± 4.2 nM. These values were similar to those required to stimulate inositol phosphate accumulation.

In rat myometrium, examination of the rank order of potencies of ET-1, ET-3, and (Thr^2)-SRTX-b, an active analog of SRTX-b (14), both in inducing inositol phosphate accumulation and in inhibiting cAMP generation indicated that both signaling pathways responded in complete agreement with the ET peptide selectivity for the ET_A receptors. Thus, ET-1 and (Thr^2)-SRTX-b stimulated inositol phosphate accumulation and inhibited cAMP accumulation with similar potencies; the EC_{50} values for (Thr^2)-SRTX-b (100 ± 8.5 nM and 40 ± 6.3 nM for inositol phosphate accumulation and inhibition of cAMP, respectively) were very close to those for ET-1 (70 ± 5.7 nM and 30 ± 4.2 nM for inositol phosphate accumulation and inhibition of cAMP, respectively). In contrast, ET-3 was much less potent that ET-1 and (Thr^2)-SRTX-b. ET-3 at 1 μM inhibited forskolin-induced cAMP accumulation by only 30%, compared with the 45% inhibition induced by 10-fold lower concentrations of ET-1 and (Thr^2)-SRTX-b. Furthermore, BQ123, which has been shown to have high selectivity for the ET_A receptor and much weaker affinity for the ET_B receptor (22, 23), dose-dependently abolished the ET-1 stimulatory effect on inositol phosphate accumulation (K_i = 70 ± 7.6 nM). Similarly, the inhibitory effect of ET-1 on cAMP accumulation was abolished by BQ123. These observations are therefore in favor of the ET_A receptor being responsible for both ET-1 effects in myometrium, i.e., stimulation of inositol phosphates and inhibition of cAMP accumulation. This interpretation was in agreement with the demonstration in rat myometrium of a single class of ET binding sites, with a pharmacolgical pattern consistent with that of the ET_A receptor (9).

Our observation that the ET_A receptor subtype triggered both inositol phosphate and cAMP responses in the myometrium did not imply a potential contribution of the inositol phosphate/Ca^{2+} pathway to the inhibitory cAMP effects. Indeed, the ET-1-mediated inhibition of cAMP content was maintained in Ca^{2+}-depleted medium containing EGTA, under conditions where the intracellular Ca^{2+} stores are virtually depleted (26, 28). Moreover, the inhibitory effect of ET-1 was abolished by pertussis toxin, indicating a G_i-mediated process. Several lines of evidence have been provided for a role of G proteins in phospholipase C activation (29), which is also the case in the myometrium. Similarly to our observations with oxytocin, muscarinic, prostaglandin, and bombesin receptors (15, 16, 30), the present data provide indirect evidence that ET_A receptors constitute an additional example of myometrial receptors coupled to phospholipase C via a pertussis toxin-insensitive G protein. It is worth mentioning that α_q/α_{11}, the pertussis toxin-insensitive G protein α subunits that have been reported to couple to phospholipase C-β (29), can be detected in different myometrial preparations.[2]

Our data have confirmed the potent contractile activity of ET-1 in rat myometrium. The rank order of potency of ET-1 > (Thr^2)-SRTX-b ≫ ET-3 is consistent with the involvement of an ET_A receptor subtype, as suggested previously by others (9, 10, 21). In the present study, this interpretation was substantiated by the ability of BQ123 to antagonize ET-1-mediated contractions. The dose-dependent agonist profile and the dose-dependent antagonist profile versus contraction were superimposable with those obtained for the inositol phosphate and cAMP responses, illustrating that both the signaling and functional events were triggered by the same subclass of ET receptors.

Another aspect demonstrated in the present study was that ET receptors were susceptible to desensitization. Thus, preexposure of myometrium to a maximal concentration of ET-1 abolished the ability of the peptide to inhibit cAMP accumulation induced by forskolin. Under these same conditions, the inositol phosphate response to a second addition of ET-1 was reduced by 40%. The reduced inositol phosphate response was linked to a change in the rate of hydrolysis of $PtdInsP_2$, as indicated by a diminished rate of accumulation of the three inositol phosphates, with the earliest attenuation being detected for $InsP_3$. It appeared that the desensitization of the ET-1 response was a receptor-mediated homologous process. Thus, under the conditions of ET-1-induced self-desensitization, there was no loss of the tissue inositol phosphate response to oxytocin or carbachol. Also, the EC_{50} and V_{max} values for ET-1 to desensitize and to stimulate the $PtdInsP_2$ response were similar, indicating that receptor occupancy was required to induce ET-1 refractoriness. Many factors could trigger ET-1 desensitization (31); these include negative regulation of ET receptor function by protein kinase C (32, 33) activated through the diacylglycerol formed concomitantly with inositol phosphate (2, 4), down-regulation of the ET receptors ((29, 34, 35), and/or phosphorylation of the ET receptors by specific kinase(s) (31).

A striking feature associated with ET-1 desensitization was that, in spite of many rinses to wash ET-1 from peptide-treated myometrium, the inositol phosphate response expressed in the presence of LiCl was still higher than the basal level. The occurrence of a persistent activity of ET-1 after removal of ET-1 from the incubation medium has been demonstrated in other

[2] Z. Tanfin, S. Lajat, and S. Harbon, Characterization and modulation of α_q/α_{11} subunits in rat myometrium during gestation, manuscript in preparation.

cell types (25, 34–36). Resink *et al.* (34) proposed that the ET-1-receptor complexes undergo internalization and that the internalized complexes account for the inability to totally wash out ET-1 and for the maintenance of ET-1 activity. In myometrium, the addition of BQ123, the ET_A-specific antagonist, removed the residual ET-1 response. It seems reasonable to propose that the occurrence of a small inositol phosphate accumulation, subsequent to ET-1 exposure and washings, resulted from the tight binding of ET-1 to its receptor, rather than from its internalization. Consistent with this interpretation was the fact that ET-3, whose affinity for the ET_A receptor was 2 orders of magnitude lower than that of ET-1, could be removed by washing (data not shown). An analogous interpretation was provided by Bousso-Mittler *et al.* (9) for the slow reversibility of ET-1 effects in terms of contraction. However, an alternative explanation that is favored in a recent report (35) could also account for the sustained response in the absence of added ligand. Those authors observed a slow recycling of internalized ET receptors in rat aorta and demonstrated that recycling receptors were fully functional in response to added ET-1. It is conceivable that the sustained response demonstrated in the present study in desensitized myometrium in the absence of added peptide could be due to some ET-1 that had been trapped in the tissues and interacted with recycled receptors. Upon further incubation in peptide-free buffer, ET-1 may be progressively released and consequently diluted in the medium. This would provide an explanation for the progressive reversibility of the sustained response observed after washing out of ET-1.

A large body of evidence emphasizes the important role of ET in the regulation of the motility of smooth muscles. Our demonstration that in rat myometrium the dual coupling of ET_A receptors to the Ca^{2+}/$PtdInsP_2$ pathway, leading to an increase in $InsP_3$ formation, and to the inhibitory branch of the adenylyl cyclase pathway, resulting in a decrease in cAMP, provides evidence that these two messengers (15–17) are major determinants of ET-1 action on the motility of rat myometrium. A paracrine role for endometrial ET-1 has been proposed (13, 37). Our findings suggest that agonist-mediated desensitization of the two ET-1 transmembrane signaling pathways after acute exposure to ET-1 represents a negative feedback mechanism regulating the functional responsiveness of the myometrium to ET-1.

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References

1. Yanagisawa, M., and T. Masaki. Molecular biology and biochemistry of the endothelins. *Trends Pharmacol. Sci.* **10**:374–378 (1989).
2. Highsmith, R. F., K. Blackburn, and D. J. Schmidt. Endothelin and calcium dynamics in vascular smooth muscle. *Annu. Rev. Physiol.* **54**:275–277 (1992).
3. Miller, R. C., J. T. Pelton, and J. P. Huggins. Endothelins: from receptors to medicine. *Trends Pharmacol. Sci.* **14**:54–60 (1993).
4. Huggins, J. P., J. T. Pelton, and R. C Miller. The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmacol. Ther.* **59**:55–123 (1993).
5. Arai, H., S. Hori, I. Aramori, H. Ohkubo, and S. Nakanishi. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature (Lond.)* **348**:730–732 (1990).
6. Sakurai, T., M. Yanagisawa, and T. Masaki. Molecular characterization of endothelin receptors. *Trends Pharmacol. Sci.* **13**:103–108 (1992).
7. Karne, S., C. K. Jayawickreme, and M. R. Lerner. Cloning and characterization of an endothelin-3 specific receptor (ET_c receptor) from *Xenopus laevis* dermal melanophores. *J. Biol. Chem.* **268**:19126–19133 (1993).
8. Aramori, I., and S. Nakanishi. Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. *J. Biol. Chem.* **267**:12468–12474 (1992).
9. Bousso-Mittler, D., Y. Kloog, Z. Wollberg, A. Bdolah, E. Kochva, and M. Sokolovsky. Functional endothelin/sarafotoxin receptors in the rat uterus. *Biochem. Biophys. Res. Commun.* **162**:952–957 (1989).
10. Calixto, J. B., and G. A. Rae. Effects of endothelins, Bay K 8644 and other oxytocics in non-pregnant and late pregnant rat isolated uterus. *Eur. J. Pharmacol.* **192**:109–116 (1991).
11. Maggi, M., G. B. Vannelli, A. Peri, M. L. Brandi, G. Fantoni, S. Giannini, C. Torrisi, V. Guardabasso, T. Barni, V. Toscano, G. Massi, and M. Serio. Immunolocalization, binding, and biological activity of endothelin in rabbit uterus: effect of ovarian steroids. *Am. J. Physiol.* **260**:E292–E305 (1991).
12. Maggi, M., G. Fantoni, A. Peri, S. Rossi, E. Baldi, A. Magini, G. Massi, and M. Serio. Oxytocin-endothelin interactions in the uterus. *Regul. Peptides* **45**:97–101 (1993).
13. Economos, K., P. C. MacDonald, and M. L. Casey. Endothelin-1 gene expression and protein biosynthesis in human endometrium: potential modulator of endometrial blood flow. *J. Clin. Endocrinol. Metab.* **74**:14–19 (1992).
14. Bdolah, A., H. Lamthanh, C. Créminon, J. Grassi, A. Ménez, Z. Wollberg, and E. Kochva. Biological activities of (Thr^2)-sarafotoxin-b, a synthetic analog of sarafotoxin-b. *Toxicon*, in press.
15. Goureau, O., Z. Tanfin, S. Marc, and S. Harbon. Diverse prostaglandin receptors activate distinct signal transduction pathways in rat myometrium. *Am. J. Physiol.* **263**:C257–C265 (1992).
16. Marc, S., D. Leiber, and S. Harbon. Fluoroaluminates mimic muscarinic- and oxytocin-receptor-mediated generation of inositol phosphates and contraction in the guinea-pig myometrium. *Biochem. J.* **255**:705–713 (1988).
17. Do Khac, L., A. Mokhtari, M. Renner, and S. Harbon. Activation of β-adrenergic receptors inhibits Ca^{2+} entry-mediated generation of inositol phosphates in the guinea pig myometrium, a cyclic AMP-independent event. *Mol. Pharmacol.* **41**:509–519 (1991).
18. Garret, N. E., and P. Garret. Rapid breakdown of diphosphoinositide and triphosphoinositide in erythrocyte membranes. *J. Cell. Physiol.* **87**:63–70 (1976).
19. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–650 (1987).
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Rae, G. A., J. B. Calixto, and P. d'Orléans-Juste. Big-endothelin-1 contracts rat isolated uterus via a phosphoramidon-sensitive endothelin ET_A receptor-mediated mechanism. *Eur. J. Pharmacol.* **240**:113–119 (1993).
22. Eguchi, S., Y. Hirata, M. Ihara, M. Yano, and F. Marumo. A novel ET_A antagonist (BQ-123) inhibits endothelin-1-induced phosphoinositide breakdown and DNA synthesis in rat vascular smooth muscle cells. *FEBS Lett.* **302**:243–246 (1992).
23. Ihara, M., K. Noguchi, T. Saeki, T. Fukuroda, S. Tsuchida, S. Kimura, T. Fukami, K. Ishidawa, M. Nishikibe, and M. Yano. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.* **50**:247–255 (1991).
24. Tanfin, Z., and S. Harbon. Heterologous regulation of cAMP responses in pregnant rat myometrium: evolution from a stimulatory to an inhibitory prostaglandin E_2 and prostacyclin effect. *Mol. Pharmacol.* **32**:249–257 (1987).
25. Gandhi, C. R., R. H. Behal, S. A. K. Harvey, T. A. Nouchi, and M. S. Olson. Hepatic effects of endothelin. *Biochem. J.* **287**:897–904 (1992).
26. Lalanne, C., C. Mironneau, J. Mironneau, and J. P. Savineau. Contractions of rat uterine smooth muscle induced by acetylcholine and angiotensin II in Ca^{2+}-free medium. *Br. J. Pharmacol.* **81**:317–326 (1984).
27. Izumi, H. Changes in the mechanical properties of the longitudinal and circular muscle tissues of the rat myometrium during gestation. *Br. J. Pharmacol.* **86**:247–257 (1985).
28. Himpens, B., and A. P. Solmyo. Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J. Physiol. (Lond.)* **395**:507–530 (1988).
29. Hepler, J. R., and A. G. Gilman. G proteins. *Trends Biochem. Sci.* **17**:383–387 (1992).
30. Amiot, F., D. Leiber, S. Marc, and S. Harbon. GRP-preferring bombesin receptors increase generation of inositol phosphates and tension in rat myometrium. *Am. J. Physiol.* **265**:C1579–C1587 (1993).
31. Wojcikiewicz, R. J. H., A. B. Tobin, and S. R. Nahorski. Desensitization of cell signalling mediated by phosphoinositidase C. *Trends Pharmacol. Sci.* **14**:279–285 (1993).
32. Resink, T. J., T. Scott-Burden, E. Weber, and F. R. Bühler. Phorbol ester promotes a sustained down-regulation of endothelin receptors and cellular

responses to endothelin in human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **166**:1213–1219 (1990).
33. Calderone, A., J. L. Rouleau, J. de Champlain, P. Bélichard, and D. J. Stewart. Regulation of the endothelin-1 transmembrane signaling pathway: the potential role of agonist-induced desensitization in the coronary artery of the rapid ventricular pacing-overdrive dog model of heart failure. *J. Mol. Cell. Cardiol.* **25**:895–903 (1993).
34. Resink, T. J., T. Scott-Burden, C. Boulanger, E. Weber, and F. R. Bühler. Internalization of endothelin by cultured human vascular smooth muscle cells: characterization and physiological significance. *Mol. Pharmacol.* **38**:244–252 (1990).
35. Marsault, R., E. Feolde, and C. Frelin. Receptor externalization determines sustained contractile responses to endothelin-1 in the rat aorta. *Am. J. Physiol.* **264**:C687–C693 (1993).
36. Yanagisawa, M., and T. Masaki. Endothelin, a novel endothelium-derived peptide. *Biochem. Pharmacol.* **38**:1877–1883 (1989).
37. Peri, A., G. B. Vannelli, G. Fantoni, S. Giannini, T. Barni, C. Orlando, M. Serio, and M. Maggi. Endothelin in rabbit uterus during pregnancy. *Am. J. Physiol.* **263**:E158–E167 (1992).

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