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Activation of Tyrosine Kinase Pathway by Vanadate in Gallbladder Smooth Muscle*

Soledad Alcón, Pedro J. Camello, Luis J. García and María J. Pozo†
Department of Physiology, University of Extremadura, 10071 Caceres, Spain

ABSTRACT. Vanadate, an inhibitor of tyrosine phosphatase activity, might induce gallbladder contraction through the stimulation of the tyrosine kinase pathway. The aim of this study was to characterize the effects of vanadate in the guinea pig gallbladder smooth muscle. Vanadate exerts contractile effects which are not mediated by neurotransmitter release. The tyrosine kinase inhibitor genistein nearly abolished vanadate contraction, suggesting that an increase in protein tyrosine phosphorylation mediates the actions of vanadate. This suggestion was confirmed by Western blot analysis. Vanadate contractions were reduced in the presence of methoxyverapamil or in Ca^{2+}-free medium, suggesting that vanadate may induce Ca^{2+} influx. Neither inactivation of the Na^+/K^+ pump nor reversal of the Na^+/Ca^{2+} exchanger can account for vanadate's actions. Vanadate contractile effects were reduced by indomethacin, as well as mepacrine, the inhibitor of phospholipase A_2, but were not affected by phospholipase C inhibitors. Neither inhibitors of diacylglycerol lipase nor protein kinase C reduced the response induced by vanadate. These data indicate that the effects of vanadate on smooth muscle are mainly mediated by protein tyrosine phosphorylation and reveal a new link between tyrosine phosphorylation and arachidonic acid metabolism in the control of gallbladder smooth muscle contraction. BIOCHEM PHARMACOL **59**;9:1077–1089, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. gallbladder motility; vanadate; protein tyrosine phosphorylation; calcium; Na^+/K^+-ATPase; arachidonic acid metabolism

Phosphorylation of tyrosine residues in signaling proteins mediates cellular proliferation [1] but is also an important mechanism in the contraction of smooth muscle [2]. Agonists such as epidermal growth factor and platelet-derivated growth factor act on tyrosine kinase receptors to induce contraction [3, 4]. In addition, certain G protein-coupled receptor agonists such as angiotensin II, vasopressin, bombesin, and endothelins may act through stimulation of non-receptor tyrosine kinases [2, 4]. Protein tyrosine phosphorylation may participate in the regulation of mechanisms that couple receptor activation to increases in $[Ca^{2+}]_i$‡ [5–7] and/or mechanisms that couple receptor activation to modulation of Ca^{2+} sensitivity to contraction [7]. Vanadate, a commonly occurring form of the trace element vanadium, is a potent inhibitor of phosphotyrosine phosphatase activity [8] and consequently increases protein tyrosine phosphorylation of several substrates [6, 9]. However, this is not the only mechanism described for the actions of vanadate. Vanadate-induced contractions have been related to inhibition of Na^+/K^+-ATPase [10, 11] and inhibition of Ca^{2+}-dependent ATPase activity [12, 13]. Although vanadate contracts several smooth muscle preparations [9, 11, 12, 14, 15], experimental data linking this contraction to tyrosine phosphorylation are very scarce [6, 9].

The effects of vanadate or its activation of the tyrosine kinase-dependent pathway in gallbladder smooth muscle contractility have not yet been explored. Thus, the purpose of this study was to determine whether vanadate had any effects on gallbladder smooth muscle and to examine the mechanisms underlying these effects.

MATERIALS AND METHODS

Dissection and Contractile Stimulation of Guinea Pig Gallbladder Smooth Muscle Strips

Gallbladders were isolated from 300–450 g male guinea pigs following cervical dislocation and exsanguination and immediately placed in cold K–HS (for composition, see *Chemicals, Reagent, and Solutions* below) at pH 7.35. The gallbladder was opened by cutting along the longitudinal axis and trimmed of any adherent liver tissue. Following washing with the nutrient solution to remove any biliary component, the mucosa was scraped off and the gallbladder was cut into strips along the longitudinal axis, each strip measuring approximately 3 × 10 mm. On average, four

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† Corresponding author: Dr. Maria J. Pozo, Department of Physiology, Nursing School, Avda Universidad s/n, 10071 Cáceres, Spain. Tel. 34-927-257450; Fax: 34-927-257451; E-mail: mjpozo@unex.es

‡ *Abbreviations:* AA, arachidonic acid; ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular calcium concentration; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; K–HS, Krebs–Henseleit solution; PLA_2, phospholipase A_2; PLC, phospholipase C; and PKC, protein kinase C.

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strips were obtained from each guinea pig gallbladder. Each strip was placed vertically in a 10-mL organ bath filled with the nutrient solution maintained at 37° and gassed with 95% O_2–5% CO_2. Isometric contractions were measured using force displacement transducers connected to a MacLab system consisting of a MacLab hardware unit and software application which run on the Macintosh computer to which the MacLab is connected. The strips were placed under an initial resting tension equivalent to 1.5-g load and allowed a 60-min period for equilibration, during which time the nutrient solution was changed every 20 min. After that period, the length of each strip was increased at a rate of 1 mm at a time until a maximal response to ACh (10 μM) was achieved. The muscle length corresponding to the optimal preload was maintained throughout the duration of the experiments.

Effect of Vanadate on Gallbladder Tone and Its Modification by Drugs and Other Interventions

After testing that no desensitization was observed in response to vanadate, a cumulative concentration–response curve for vanadate was made (10 μM–3 mM). In some tissues, a second concentration–response curve was obtained following a wash period of 60 min. The effects of either atropine, D-609, genistein, GF109203X, indomethacin, mepacrine, mehoxyverapamil, phentolamine, ryanodine, tetrodotoxin (TTX), tyrphostin B44, or U-73122 on vanadate-induced responses were studied by addition of these agents, in known concentrations, 20 min before the second curve was obtained.

The response of gallbladder strips to vanadate was also assayed in preparations bathed in modified solutions (for composition, see *Chemicals*, *Reagents*, and *Solutions*): Ca^{2+}-free K–HS containing EGTA (1 mM), Ca^{2+}-free Sr^{2+} K–HS, K^+-free K–HS, and Na^+-deficient (25 mM) K–HS. In the case of the Ca^{2+}-free solution, single doses of vanadate were tested immediately after the substitution of Ca^{2+}-free K–HS by normal K–HS to avoid Ca^{2+} store depletion. Test tissues were exposed to the Ca^{2+}-free Sr^{2+} K–HS for 30 min before and throughout the vanadate challenge. To assess the effect of vanadate on K^+-induced relaxation, the strips were incubated for 30 min in K^+-free solution; then, for test tissues, the bath solution was exchanged for K^+-free solution containing vanadate (100 μM) or ouabain (10 μM). Fifteen minutes later, test tissues were challenged with KCl (30 mM). Control tissues were subjected to the same protocol, but were not exposed to vanadate or ouabain. In the case of the Na^+-deficient solution, the preincubation time was increased to 60 min.

Immunochemical Determination of Protein Tyrosine Phosphorylation

To determine the influence of sodium vanadate on protein tyrosine phosphorylation, the muscle strips were prepared and treated in an identical manner as in the contraction studies. The strips were removed from the organ bath immediately before reaching maximal contraction in response to 100 μM of vanadate (a concentration close to its EC_{50}) either in the presence or absence of the tyrosine kinase blockers genistein (100 μM) and tyrophostin B44 (100 μM), and then clamped with nitrogen-cooled forceps. The muscle strips were later ground in a liquid nitrogen-cooled mortar and pestle, homogenized in lysis solution (LS; for composition, see *Chemicals*, *Reagents*, *and Solutions*) using a homogenizer (OMNI International), and then sonicated for 5 sec. Lysates were centrifuged at 10,000 *g* for 15 min. Protein concentration was measured by the Bio-Rad protein assay reagent using BSA as standard and the volume adjusted so that 1-mL aliquots of lysates contained the same amount of protein (200 μg/mL).

The level of tyrosine phosphorylation was determined as follows: aliquots were incubated with 4 μg of antiphosphotyrosine monoclonal antibody (PY20), 4 μg of goat anti-mouse immunoglobulin G (IgG), and 30 μL of protein A-agarose overnight at 4°. The immunoprecipitates were washed three times with PBS (for composition, see *Chemicals*, *Reagents*, *and Solutions*). Antiphosphotyrosine immunoprecipitates were fractionated by SDS–PAGE with a NOVEX system using 10% polyacrylamide gels. Proteins with molecular masses higher than 30 kDa were transferred to 0.20-μm pore size nitrocellulose membranes. Membranes were blocked overnight at 4° using blotto solution (BS; for composition, see *Chemicals*, *Reagents*, *and Solutions*) and incubated for 3 hr at 25° with 1 μg/mL antiphosphotyrosine monoclonal antibody ($4G_{10}$, UBI). After incubation with the primary antibody, membranes were washed twice for 10 min with blotto solution and incubated for 1 hr at 25° with anti-mouse IgG–horseradish peroxidase conjugate. The membranes were finally washed twice for 10 min with blotto solution and twice for 10 min with washing solution (WS; for composition, see *Chemicals*, *Reagents*, *and Solutions*), incubated with enhanced chemiluminescence detection reagents (ECL) for 60 sec, and exposed to Hyperfilm ECL.

Chemicals, Reagents, and Solutions

Drug concentrations are expressed as final bath concentrations of active species. Drugs were obtained from the following sources: acetylcholine chloride, CCCP, EGTA, genistein, indomethacin, (6-chloro-9-(4-diethylamino)-1-methyl-butyl)amino-2-methoxyacridine-(mepacrine), methoxyverapamil hydrochloride, ouabain octahydrate, sodium orthovanadate, and tetrodotoxin were from Sigma Chemical Co., atropine sulfate monohydrate was from Across, and D-609, GF109203X, RHC 80267, ryanodine, tyrphostin B44, and U-73122 were from Calbiochem. Other chemicals used were of analytical grade (Panreac). Stock solutions of genistein, tyrphostin, and U-73122 were prepared in DMSO. The solutions were diluted so that the final concentration of DMSO in the organ bath was ≤0.1% v/v. This concentration of solvent did not itself affect the

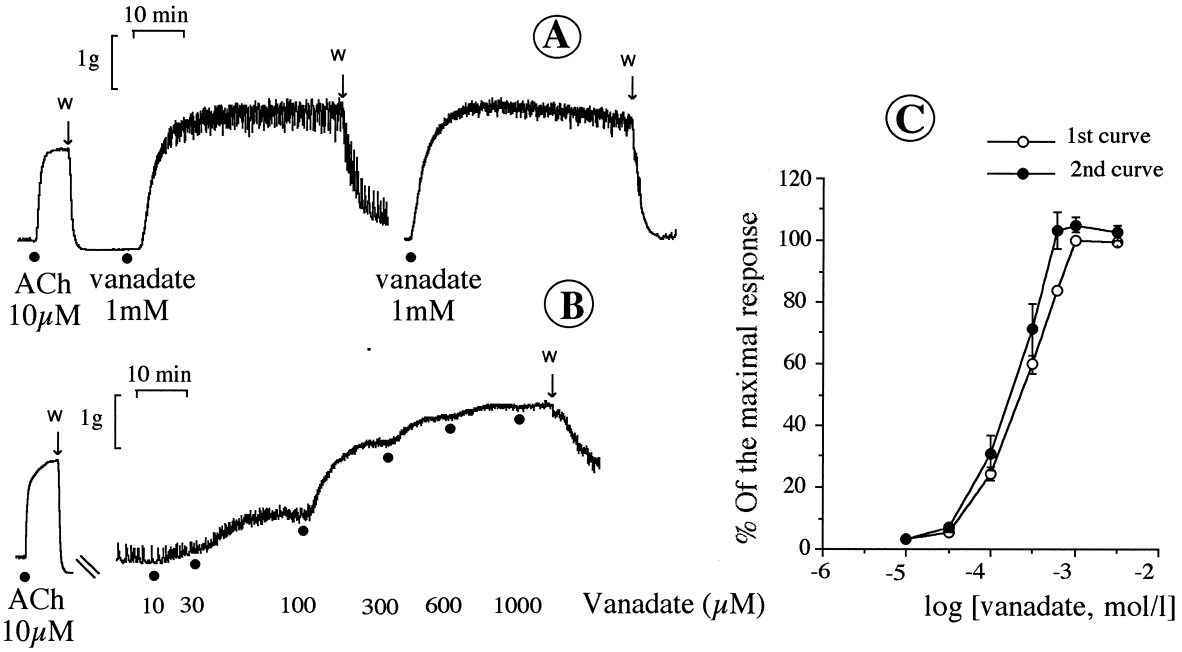

FIG. 1. Contractile effects of vanadate in guinea pig gallbladder strips. (A) Original recording showing the response of the tissue to single doses of ACh (10 μM) and vanadate (1 mM). Maximal tension in response to vanadate was achieved after 8–10 min of challenge and was stable for up to 30 min. The response to vanadate was reversible following washout, and reproducible contractions were observed at 30-min intervals. (B) When the gallbladder strip was exposed to cumulative concentrations of vanadate, concentration-dependent contractions were observed. (C) Log concentration–response curves for vanadate in guinea pig gallbladder strips. The second curve was obtained following a wash period of 60 min. Note that there are not significant differences between the two curves (by ANOVA), showing that no desensitization of the tissue had occurred. Abscissa scale: molar concentration of vanadate on a log scale. Ordinate scale: tension developed expressed as a percentage of that evoked by 1 mM vanadate (in the first curve). Data points indicate means of values from 6 experiments. Vertical lines indicate SE of the mean. Traces in A and B are typical of six such experiments. W: washout.

mechanical activity of the tissue. Genistein and tyrphostin B44 were protected from light exposure.

The composition (mM) of the K–HS was: NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, and D-glucose 11.5. The Ca^{2+}-free K–HS was prepared by substituting EGTA (1 mM) for $CaCl_2$. The K^+-free K–HS was prepared by removing KCl from the K–HS and replacing KH_2PO_4 with NaH_2PO_4. The Na^+-deficient (25 mM) K–HS was prepared by isosmotic replacement of NaCl by sucrose. The Ca^{2+}-free Sr^{2+} K–HS was prepared by equimolar substitution of Sr^{2+} by Ca^{2+}. The composition of the solutions used in the immunochemical determination of protein tyrosine phosphorylation was: lysis solution (LS): 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% (w/v) NaN_3, 1 mM EGTA, 0.4 mM EDTA, 2.5 μg/mL aprotinin, 25 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM Na_3VO_4. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 5.62 mM Na_2HPO_4, 1.09 mM NaH_2PO_4, 1.47 mM KH_2PO_4, and 0.2 mM Na_3VO_4. Blotto solution (BS): 5% non-fat dried milk in a solution containing 50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20, and 0.02% NaN_3. Washing solution (WS): 50 mM Tris/HCl pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20, and 0.02% NaN_3.

Statistics

Contractile responses are expressed in absolute values (g) and/or as percentages of the maximal response elicited by vanadate (1 mM) or ACh (10 μM). Each concentration curve was analyzed to evaluate the concentration producing 50% of maximal response (EC_{50}) and the maximum asymptote of the curve (E_{max}) according to Tallarida and Murray [16] by using a computer program. EC_{50} is also expressed as $-\log(EC_{50})$ (pD_2). All values are presented as means ± SE of the mean. Statistical evaluation was performed by two-way analysis of variance (ANOVA) or by using Student's *t*-test (two-tailed) as appropriate. Differences were considered significant when $P < 0.05$.

RESULTS

Contraction of Isolated Gallbladder Preparation by Vanadate

Isolated gallbladder strips contracted in response to increasing concentrations of vanadate (10 μM–3 mM). Figure 1A shows examples of isometric tension responses of the tissue to a single (1 mM) concentration of vanadate. In this preparation, the onset of tension development in response to vanadate occurred within 10–30 sec of challenge and

TABLE 1. Effect of either phentolamine, atropine, or tetrodotoxin on guinea pig gallbladder strip contractions to vanadate

	Phentolamine (36 μM)		Atropine (1 μM)		Tetrodotoxin (1 μM)	
Vanadate (μM)	without	with	without	with	without	with
10	5.4 ± 2.7	5 ± 0.6	11.7 ± 4.5	9.7 ± 3.5	10.9 ± 1.3	11.3 ± 3.7
30	5.1 ± 2.3	9.2 ± 0.7	29.7 ± 8.6	18.0 ± 5.7	28.2 ± 3.4	18.5 ± 5.0
100	29.6 ± 7.9	44.8 ± 6.2	58.0 ± 9.7	47.0 ± 8.3	64.1 ± 2.3	50.9 ± 7.3
300	63.8 ± 6.7	75.7 ± 8.3	70.3 ± 6.8	62.5 ± 7.1	80.1 ± 1.8	72.8 ± 8.3
600	86.1 ± 3.9	90.5 ± 12.3	90.6 ± 4.4	82.5 ± 7.8	94.5 ± 0.7	89.4 ± 9.7
1000	100 ± 0	96.6 ± 14.1	100 ± 0	89.8 ± 9.0	100 ± 0	94.1 ± 9.6

Results expressed as a percent of maximal response (mean ± SEM), with experiments performed in 6 muscle strips.

maximal tension was achieved after 8–10 min. This tension could be maintained for up to 30 min, showing that vanadate can exert prolonged effects on the tone of gallbladder smooth muscle. Responses to vanadate were reversible following washout, and reproducible contractions could be elicited at 30-min intervals (Fig. 1A). In view of the characteristics of the responses to vanadate, it was possible to make sequential measurements of the contractile responses to increasing concentrations of vanadate. When strips were challenged with cumulative concentrations of vanadate, concentration-dependent tension development was observed (Fig. 1, B and C). The maximal effect of vanadate was 2.11 ± 0.29 g, which was equivalent to 201.58 ± 32.42% of the response to ACh (10 μM). This maximal effect was achieved at the concentration of 1 mM, and higher concentrations of vanadate induced similar responses. The pD_2 for vanadate was 4.11 ± 0.09. When two concentration–response curves to vanadate were made, no desensitization of the tissue was observed (Fig. 1C); therefore, to study the effects of different compounds on vanadate-induced contractions, two dose–response curves were done sequentially.

Role of Endogenous Neurotransmitters in Vanadate-Induced Contractions

Vanadate did not appear to act via direct or indirect stimulation of muscarinic or alpha-adrenoceptors located on the gallbladder smooth muscle membrane, because the response to all concentrations of vanadate assayed was unaltered by tissue pretreatment (20 min before the second vanadate challenge) with 1 μM atropine or 36 μM phentolamine (Table 1). Since vanadate-induced contractions were not susceptible to neural blockade with tetrodotoxin (1 μM) (Table 1), the release of neurotransmitters from intrinsic nerves was not involved in the action of vanadate in the gallbladder.

Role of Phosphotyrosine Phosphorylation in Vanadate Effects

In view of the known ability of vanadate to inhibit protein tyrosine phosphatase [8], potentiation of the actions of tissue tyrosine kinases could be expected. Thus, we wanted to determine if different tyrosine kinase inhibitors would affect the contractile action of vanadate. Preincubation of the strips with 100 μM genistein, a naturally occurring flavonoid which potently inhibits several tyrosine kinases [17], almost abolished the contraction evoked by vanadate. The maximal effect of vanadate was 2.01 ± 0.27 g before and 0.29 ± 0.09 g after genistein ($P < 0.01$, N = 6) (Fig. 2B). In contrast, tyrphostin B44 (100 μM), a tyrosine kinase inhibitor structurally unrelated to genistein [18], did not decrease the vanadate maximal effect (Fig. 2D), but caused rather a significant leftward shift of the vanadate concentration–response curve (EC_{50} of 111.22 ± 17.6 μM vs 51.19 ± 8.95 μM, $P < 0.05$ by Student's *t*-test).

To determine if vanadate increased tyrosine phosphorylation, we analyzed, by a Western blotting procedure, the phosphotyrosyl protein content of the gallbladder muscle which had been contracted isometrically with 100 μM vanadate for 10 min either in the absence or presence of either genistein or tyrphostin B44 (Fig. 3). Vanadate-induced contractions were associated with increased tyrosine phosphorylation of several substrates, including proteins with molecular masses of 175, 130, and 75 kDa that were also visualized in untreated samples (Fig. 3, lanes 1C and 2C). When tissues were treated with vanadate in the presence of the same concentration of genistein (100 μM) that blocked vanadate-induced contraction, the phosphoprotein signals on the immunoblot remained at control levels (Fig. 3, lane 1D). Genistein alone slightly reduced basal tyrosine phosphorylation (Fig. 3, lane 1B). Tyrphostin B44, like genistein, decreased basal levels of tyrosine phosphorylation (Fig. 3, lane 2B). Surprisingly, and unlike the effects seen with genistein (Fig. 3, lane 1D), tyrphostin B44 pretreatment greatly enhanced tyrosine phosphorylation in response to vanadate (Fig. 3, lane 2D).

Role of Calcium in Vanadate-Induced Responses

To study whether vanadate influenced Ca^{2+} influx involved in the excitation–contraction coupling, the effect of methoxyverapamil (D-600), an antagonist of voltage-gated Ca^{2+} channels, on responses to vanadate was investigated. The addition of 10 μM methoxyverapamil significantly reduced the contractile responses of vanadate to all con-

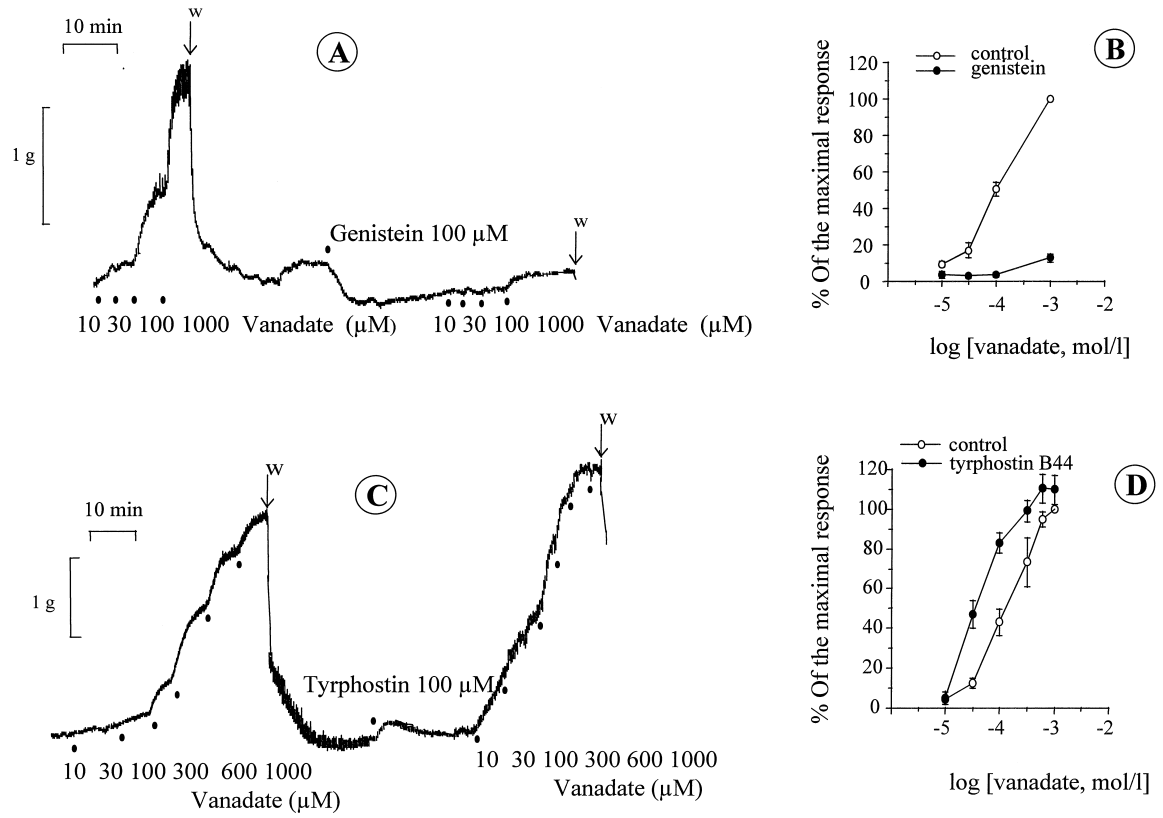

FIG. 2. Effects of tyrosine kinase inhibitors on the contractile response to vanadate. A and C are original traces showing the typical responses of the tissue to vanadate in the absence and presence of the tyrosine kinase inhibitors. Traces are typical of 6 such different experiments. W: washout. (B) Preincubation of the strips with 100 μM genistein significantly reduced the contractions evoked by vanadate ($P < 0.01$ by ANOVA). (D) In contrast, in the presence of tyrphostin B44, the responses to vanadate were higher than in the control test. Abcissa scales: molar concentration of vanadate on a log scale. Ordinate scales: tension developed expressed as a percentage of that evoked by 1 mM vanadate alone. Data points indicate means from 6 experiments and vertical lines show SE of the mean.

centrations assayed. The maximal response of the gallbladder to vanadate was significantly reduced by 47.82 ± 3.23% ($P < 0.01$) (Fig. 4A). This concentration of D-600 completely relaxed preparations that had been contracted with 60 mM KCl (2.06 ± 0.25 g vs 0.07 ± 0.01 g, $P < 0.01$) in 16.67 ± 1.47 min.

Because intracellular calcium stores in the gallbladder muscle are quickly depleted in the absence of extracellular calcium [19], the strips were suddenly bathed with zero calcium K–H. Contractions in response to vanadate added immediately after the bathing solution was replaced with Ca^{2+}-free K–HS containing EGTA (1 mM) were nearly abolished when the lower concentrations (10, 30, and 100 μM) were assayed and largely reduced when the higher ones (0.3, 0.6, and 1 mM) were tested (19.35 ± 5.49%, 18.97 ± 8.36%, and 25.99 ± 14.63% compared to responses in normal K–HS) (Fig. 4B). Moreover, in contrast to contractions in Ca^{2+} K–HS, which could be maintained for up to 30 min, the contractions in calcium-free medium rapidly declined and disappeared within 8–10 min.

To determine whether the residual response to higher concentrations of vanadate in the absence of extracellular calcium was due to the release of calcium from intracellular stores, an equimolar substitution of strontium for calcium in the bathing solution was made. Previous studies have shown that strontium can replace extracellular calcium but cannot substitute for stored calcium. Although strontium is incorporated in the endoplasmic reticulum and displaces calcium from high-affinity binding sites, it cannot be easily released from stored sites [20, 21]. Thus, these replacement experiments should be useful in establishing whether an agonist has a requirement for intracellular calcium in the contractile response [19]. In our preparation, strontium slightly increased the basal tension, caused a marked increase in phasic activity, and failed to affect the response to 60 mM KCl (137.47 ± 24.35% vs 123.95 ± 22.5% of the response to 10 μM ACh). The responses to lower concentrations of vanadate were the same as in normal K–HS (Fig. 4C), but when the higher ones were tested (0.3, 0.6, and 1 mM), a slight but significant reduction was observed ($P < 0.05$), $P < 0.01$, and $P < 0.01$, by Student's t-test, respectively, which is in agreement with the residual response observed in presence of Ca^{2+}-free K–HS.

Ryanodine, a naturally occurring alkaloid which reduces the release of calcium from sarcoplasmic reticulum by interacting specifically with some (ryanodine-sensitive)

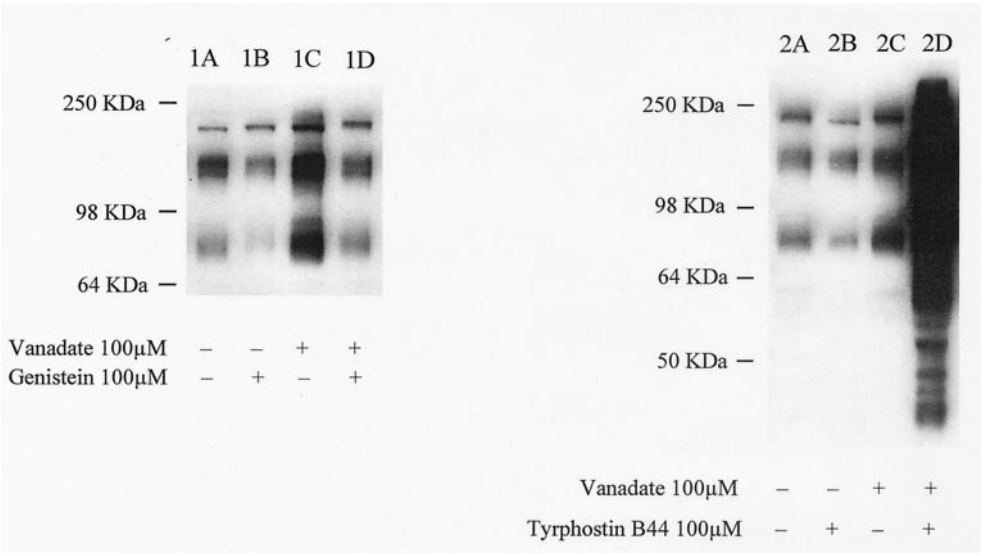

FIG. 3. Western blot analysis of phosphotyrosyl proteins in control and vanadate-treated tissues. Gallbladder strips were recovered from the organ bath, as described in Methods, and processed for Western blot analysis. Tissues were either untreated (lanes 1A and 2A) or were exposed to vanadate either in the absence (lanes 1C and 2C) or presence of genistein (100 μM, lane 1D) or tyrphostin B44 (100 μM, lane 2D). The effects of the tyrosine kinase inhibitors on basal phosphorylation were also tested (lanes 1B and 2B). Note that vanadate increased protein tyrosine phosphorylation, which was reversed by pretreatment with genistein. However, pretreatment with tyrphostin B44 induced an unexpected huge increase in phosphotyrosyl phosphorylation. These blottograms are typical of 6 such different experiments.

calcium channels [22], affected neither the basal tone nor the contractile effects of vanadate (Fig. 4D).

Role of Membrane Transport Mechanisms in the Contractile Action of Vanadate

Since vanadate is known to be a potent inhibitor of Na^+/K^+-ATPase in a number of tissues [10] and the resultant depolarization could account for the vanadate-induced contractions, gallbladder strips were incubated in a K^+-free solution. Exposure of the tissue to K^+-free medium caused, in most of the strips (N = 10), an initial contraction followed by a decrease in the baseline tension, which remained below the pre-exposure level 30 min later (Fig. 5). However, in other strips (N = 5), K^+-free medium induced a transitory relaxation which was followed by a slowly developing contraction. Both vanadate (100 μM) and ouabain (10 μM) induced contractions in K^+-free medium, and no significant differences were measured between vanadate responses in normal K–HS and K^+-free K–HS (63.43 ± 4.34% vs 70.62 ± 8.7% of the response to 10 μM ACh). When control strips which had been exposed to K^+-free medium were challenged with 30 mM KCl, an initial relaxation was observed prior to the contractile response (Fig. 5A), but when the strips had been pretreated with ouabain (Fig. 5B) or vanadate (Fig. 5C), two types of responses were observed. Ouabain pretreatment caused only contraction (Fig. 5B), but when the strips were pretreated with vanadate, the response was similar to the control tissues and the initial relaxation was observed (Fig. 5C).

Since contraction to vanadate may result secondarily from inhibition of Na^+/Ca^{2+} exchange across the plasma membrane, the strips were exposed to a reduced Na^+ K–HS. Under these conditions, a marked contraction occurred and after reaching a maximum, which was equivalent to 77.10 ± 6.72% of the response to 10 μM ACh (N = 16), the tension returned to the pre-existing baseline within 7–10 min. As shown in Fig. 6, when vanadate is tested in a Na^+-deficient K–HS, the contractile response is the same as in normal K–HS.

Vanadate and phosphate compete in a number of biological systems, e.g. ATP synthesis, which could lead to mitochondrial uncoupling. To test this hypothesis, we exposed the gallbladder strips to the oxidative phosphorylation uncoupler CCCP. A sustained relaxation of the muscle strips with disappearance of their phasic activity was observed after exposure to CCCP (10 μM) (Fig. 7A). Furthermore, CCCP significantly inhibited the vanadate (1 mM)-induced contraction by 74.55 ± 4.58% ($P < 0.01$, by Student's *t*-test) (Fig. 7).

Intracellular Pathways Stimulated by Vanadate

Since it has been reported that the contractile actions of agents that increase protein tyrosine phosphorylation in gastric longitudinal muscle are entirely dependent on the production of prostanoids [23], we studied if the actions of vanadate on the gallbladder were sensitive to indomethacin (an inhibitor of the cyclooxygenase pathway). As shown in Fig. 8A, indomethacin significantly reduced the responses to vanadate ($P < 0.01$ by ANOVA). The source of AA for

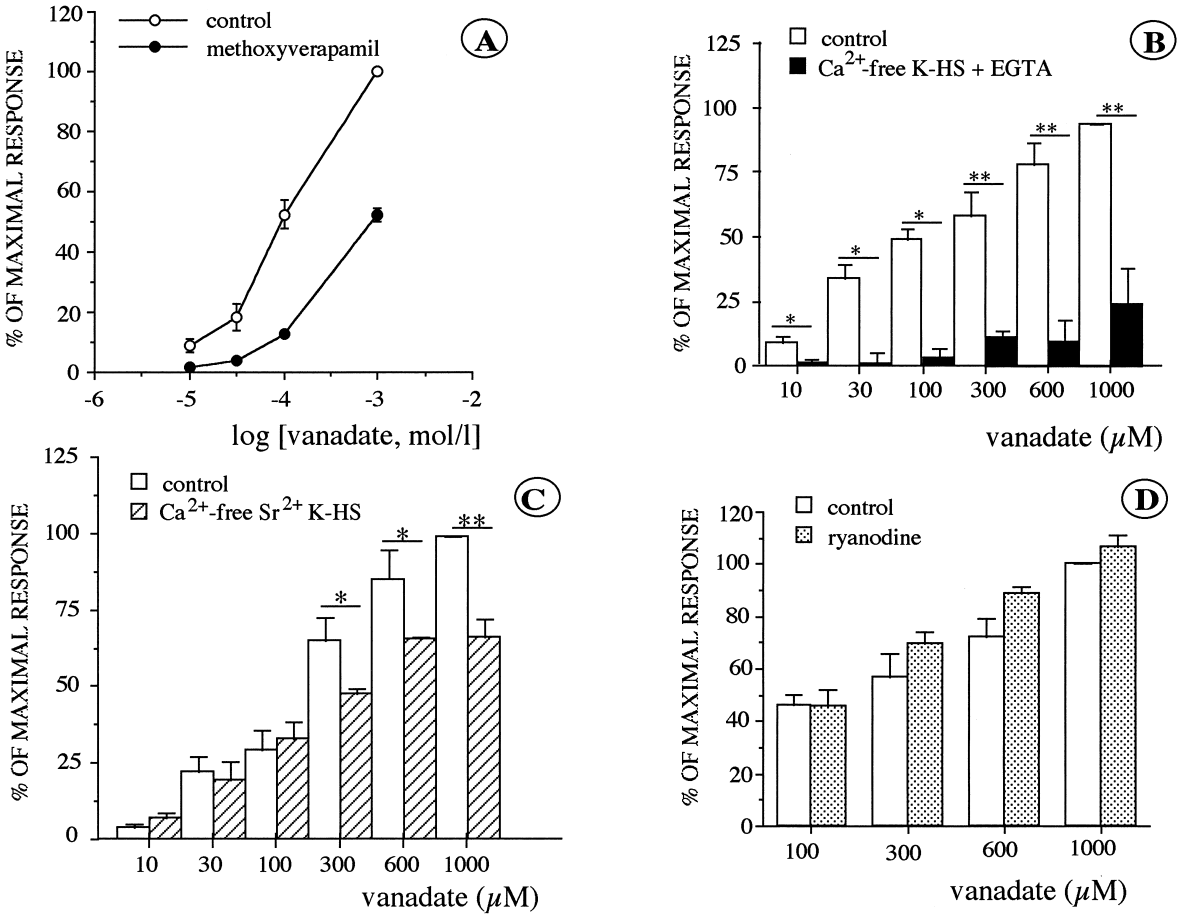

FIG. 4. Role of calcium in vanadate-induced gallbladder contraction. (A) Inhibitory effects of methoxyverapamil, an L-type calcium channel blocker, on the contractile response to vanadate. Log concentration–response curve for the contractile action of vanadate alone and after a 20-min incubation with 10 μM methoxyverapamil. Abcissa scale: molar concentration of vanadate on a log scale. Ordinate scale: tension developed expressed as a percentage of that evoked by 1 mM vanadate in the absence of methoxyverapamil. All responses to vanadate in the presence of methoxyverapamil were smaller than those observed in the control tissues ($P < 0.01$ by ANOVA). (B) Effect of the sudden removal of extracellular Ca^{2+} on the contractile responses to single doses of vanadate (10 μM–1 mM). Vanadate was tested immediately after the removal to avoid Ca^{2+} store depletion. The contractile responses to all concentrations of vanadate were significantly reduced in the absence of extracellular calcium (*$P < 0.05$, **$P < 0.01$ by paired Student's *t*-test). (C) Effect of the equimolar substitution of strontium for calcium in the bathing solution. After testing the vanadate response in normal K–HS, tissues were exposed to the Ca^{2+}-free Sr^{2+} K–HS for 30 min before the same concentration of vanadate was tested. Note that only the responses to higher concentrations of vanadate (0.3, 0.6, and 1 mM) were significantly reduced (*$P < 0.05$, **$P < 0.01$ by paired Student's *t*-test). (D) Effect of ryanodine (10 μM) on the contractile responses to vanadate. Ryanodine, a naturally occurring alkaloid which blocks the release of calcium from sarcoplamic reticulum, did not affect the contractile effect of vanadate. Results are expressed as a percentage of the tissue response to 1 mM vanadate in normal K–HS. Each data point/histogram shows values for the mean ± SE of 6 experiments.

the synthesis of prostaglandin(s) could result either from the activation of PLA_2 or from the sequential action of other phospholipases (e.g., PLC), followed by metabolism of the released diacylglycerol by diacylglycerol lipase (DG-lipase). To distinguish between these two alternatives, we used the DGlipase inhibitor RHC 80267, which potently and selectively inhibits DGlipase in a variety of tissues but causes little or no inhibition of other lipases [24], and the PLA_2 inhibitor mepacrine [25]. Mepacrine at 100 μM significantly ($P < 0.05$ by ANOVA) reduced vanadate-induced contractions (Fig. 8B). However, RHC 80267 failed to reduce vanadate responses, and an increase in the response to the higher concentrations of vanadate was even observed (Fig. 8C). Pretreatment of the tissue with either 1 or 10 μM GFX109203, a specific inhibitor of the PKC, had no effect on the vanadate response (Fig. 8D). The lower dose of GFX109203 was able to reduce the response to a known activator of PKC such as cholecystokinin (CCK) (data not shown). As could be expected taking into account our above results, a combination of the phosphatidylcholine-dependent phospholipase C (PC–PLC) inhibitor D-609 (100 μM) and the phosphatidyl-inositol-dependent phospholipase C (PI–PLC) inhibitor U-73122 (8.6 μM) had no effect on vanadate-induced contractions, although this combination was able to significantly reduce the CCK-evoked contractile response (data not shown).

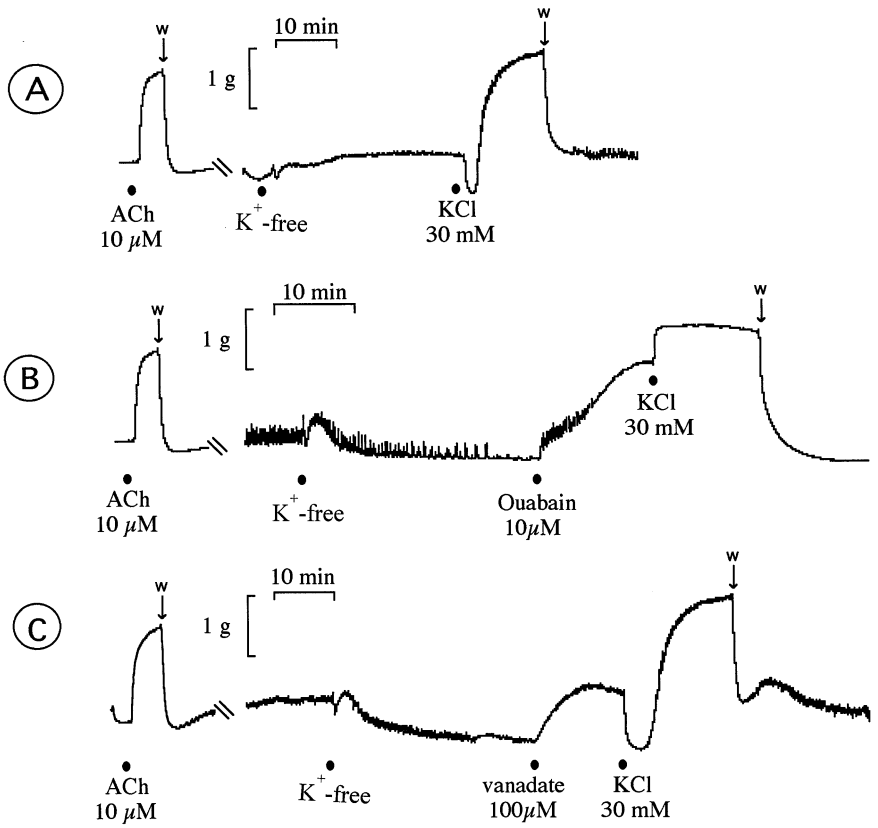

FIG. 5. Lack of effect of vanadate on Na^+/K^+-ATPase activity. (A) Exposure to K^+-free medium induced an inhibition of Na^+/K^+-ATPase which was reversed by addition of KCl. The initial relaxation in response to 30 mM KCl was due to reactivation of the pump, which was followed by the normal contractile response to KCl. (B) When ouabain was added to the K^+-free bath solution, an increase in the tone was observed. The addition of KCl caused only a contractile response without the initial relaxation, proving that the Na^+/K^+-ATPase was blocked. (C) When the same protocol was followed for vanadate, the initial relaxation in response to KCl addition was observed, proving that vanadate does not inhibit Na^+/K^+-ATPase. Traces in A, B, and C are typical of six such different experiments. W: washout.

DISCUSSION

The results of the present experiments demonstrate that vanadate exerts prolonged contractile effects on the tone of the gallbladder which are not mediated by release of neurotransmitter from the intrinsic gallbladder plexus or by direct or indirect activation of muscarinic and adrenergic receptors, similar to previous reports in other smooth muscle types [9, 11, 12, 13].

Role of Phosphotyrosine Phosphorylation in Vanadate Effects

Tyrosine phosphorylation is an important mechanism for regulation of smooth muscle contraction [2]. Since tyrosine phosphorylation depends on the balance between the activity of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP), it has been postulated that inhibition of tyrosine phosphatase activity should increase protein tyrosine phosphorylation in smooth muscle and thereby induce contraction [7]. Our results are entirely in agreement with this hypothesis, because vanadate caused a contraction that is associated with an increase in tyrosine phosphorylation of several substrates. Preincubation with genistein, a tyrosine kinase inhibitor, almost completely inhibited both vanadate-induced contractions and vanadate-induced tyrosine phosphorylation, which demonstrates that protein tyrosine phosphorylation is the key regulatory factor mediating vanadate-induced contractions. The results of this study indicate that steady-state (non-stimulated) kinase and phosphatase activities (and therefore phosphorylation and dephosphorylation) occur in gallbladder muscle cells, and that regulation of this balance may be a physiological mechanism for altering gallbladder tone.

Our work supports previous studies demonstrating that vanadate-induced contractions in gastrointestinal and vascular preparations are associated with enhanced protein tyrosine phosphorylation [7, 9, 26]. However, Zhou *et al.* [11] concluded that tyrosine phosphorylation is not involved in the contractile response induced by vanadate in rat aorta, and Cortijo *et al.* [12] suggested that protein tyrosine phosphorylation is only of limited importance in mediating the spasmogenic effects of vanadate in human bronchus. The unexpected increase in tyrosine phosphorylations induced by vanadate in the presence of tyrphostin B44 could be due to the phosphorylation of proteins related

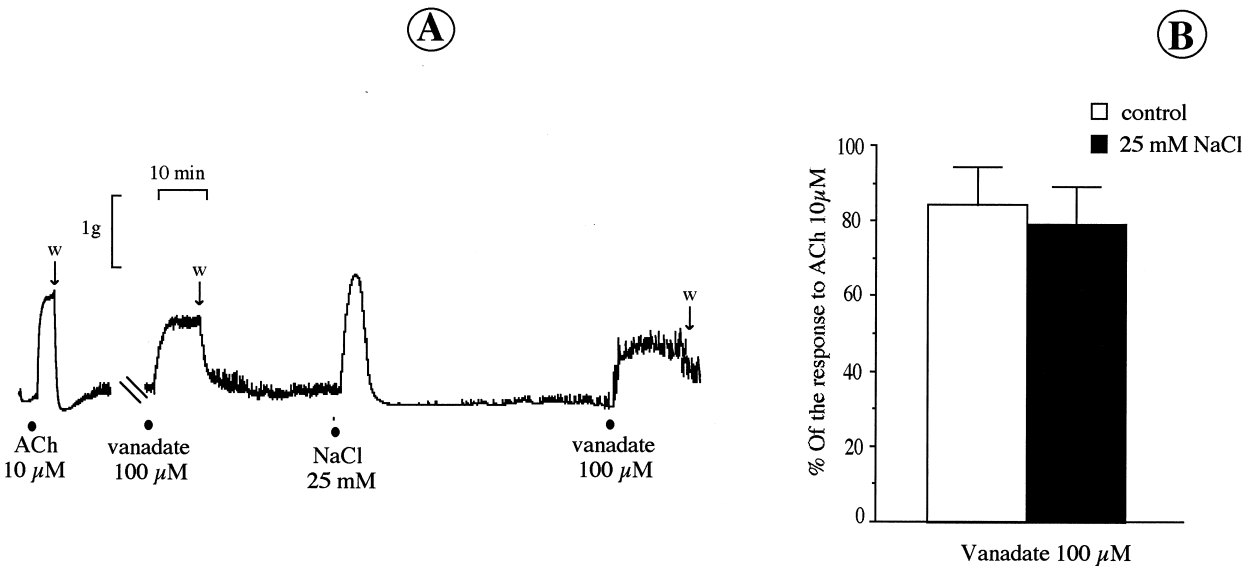

FIG. 6. Effects of Na^+-deficient medium in vanadate-induced contraction. (A) After testing the response to vanadate (100 μM) in normal K–HS, a substitution of Na^+-deficient (25 mM) K–HS for normal K–HS was made. This manoeuvre induced a transitory contraction which returned to the pre-existing baseline within 7–10 min. When the response to vanadate was assayed in this medium, the contraction recorded was similar to that obtained in normal K–HS. The trace is typical of 6 such different experiments. W: washout. (B) Effect of the Na^+-deficient (25 mM) K–HS on the vanadate-induced response following the above protocol. Results are expressed as a percentage of the tissue response to 10 μM ACh. Each histogram shows values for the mean ± SE of 6 experiments.

to the contractile mechanism, although phosphorylation of proteins unrelated to this process is likely to occur considering the huge phosphorylation described. This potentiation may reflect complex and reciprocal regulatory relationships between protein tyrosine kinases and tyrosine phosphatases [27, 28]. The inefficacy of tyrphostin B44 in reducing vanadate-induced phosphorylation is in agreement with a recent study showing that tyrphostin 47 did not inhibit an [arginine^8]-vasopressin (AVP)-induced [Ca^{2+}] increase, but did strongly increase vanadate-induced tyrosine phosphorylation [29]. Since Src-related kinases are negatively regulated by phosphorylation of specific tyrosine residues, the inhibition of tyrosine kinase activity by these tyrphostin compounds could enhance the activity of these proteins, which would be potentiated in the presence of phosphatase inhibitors such as vanadate. This hypothesis is

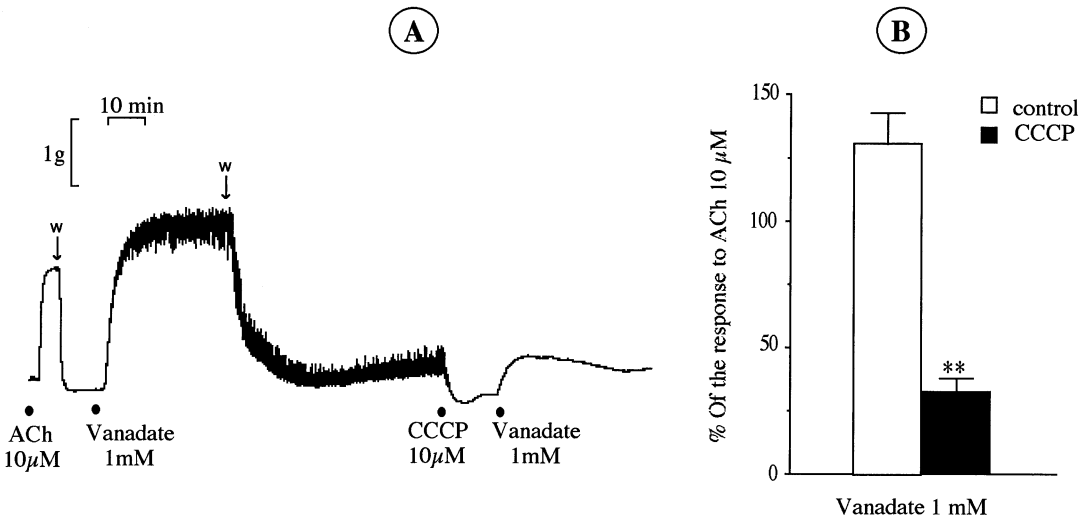

FIG. 7. Effects of CCCP in vanadate-induced contractions. (A) After the response to 10 μM ACh was tested, the response to vanadate (1 mM) was studied in the absence and presence of 10 μM CCCP, an oxidative phosphorylation uncoupler. CCCP decreased the basal tension, abolished the phasic activity, and reduced the response to vanadate. The trace is typical of 6 such different experiments. W: washout. (B) Effect of CCCP on the vanadate-induced response following the above protocol. Results are expressed as a percentage of the tissue response to 10 μM ACh. Each histogram shows values for the mean ± SE of 6 experiments. ($P < 0.01$ by paired Student's *t*-test).**

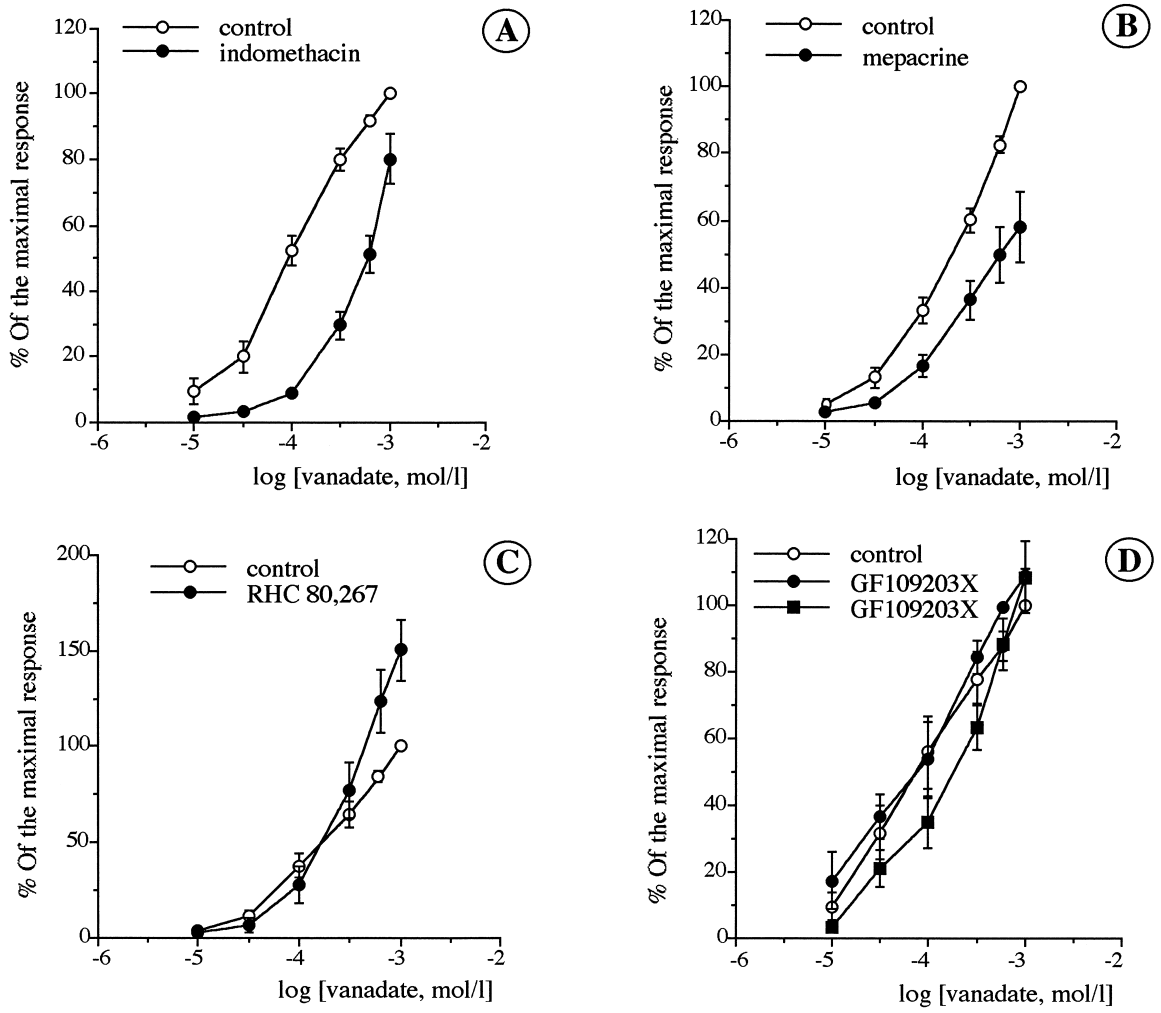

FIG. 8. Intracellular pathways stimulated by vanadate. (A) Effects of pretreatment with 10 μM indomethacin on vanadate contractile responses. All responses to vanadate in the presence of indomethacin were significantly smaller than those observed in control tissues ($P < 0.01$ by ANOVA). (B) Effects of pretreatment with the PLA_2 inhibitor mepacrine (100 μM) on the contractile response to vanadate. The responses to vanadate in the presence of mepacrine were significantly smaller than those observed in control tissues ($P < 0.05$ by ANOVA). (C) Effects of pretreatment with the diacylglycerol lipase inhibitor RHC 80267 (40 μM) on vanadate-induced contractions. RHC 80267 failed to reduce vanadate responses, and an increase in the response to higher doses of vanadate was even observed. (D) Effects of pretreatment with the PKC inhibitor GF109203X (1 and 10 μM, closed circles and squares, respectively) on vanadate-induced contractions. Neither of the GF109203X concentrations tested significantly reduced the vanadate response. Abcissa scales: molar concentration of vanadate on a log scale. Ordinate scales: tension developed expressed as a percentage of that evoked by 1 mM vanadate in normal K–HS. Data points indicate means from 6 experiments and vertical lines show SE of the mean.

supported by the different mechanisms of action reported for genistein and tyrphostins. Whereas genistein inhibits tyrosine kinase activity by interacting with the ATP-binding site [17], tyrphostins inhibit enzymatic activity by interacting with the phosphotyrosine substrate-binding site [18].

Role of Calcium in Vanadate-Induced Responses

The tyrosine-phosphorylated substrates might increase the conductance of Ca^{2+} channels in the sarcolemma and permit influx of extracellular Ca^{2+}. Such an influx could also promote release of intracellular Ca^{2+} through calcium-induced calcium release [2, 30]. This could explain the dependence of extra- and intracellular calcium shown for vanadate-induced contraction in this study. Vanadate-induced contractions of the guinea pig gallbladder require calcium influx, although calcium mobilization from intracellular stores can account for the effects of the higher concentrations of vanadate tested in this study. This conclusion is based on the following experimental observations: the use of methoxyverapamil, an L-type calcium channel blocker, reduced the contractile response to vanadate. Further, the contraction of gallbladder strips was abolished in Ca^{2+}-free medium containing EGTA (1 mM) when the lower concentrations of vanadate were tested and was reduced at higher concentrations. These results are in agreement with those obtained when calcium substitution

with strontium was made. That manoeuvre reduced the contractile response only to the higher, but not the lower, concentrations of vanadate. These findings indicate that vanadate contractions mainly depend on extracellular Ca^{2+}.

This extracellular calcium requirement for vanadate action is in agreement with previous studies that demonstrated that vanadate-induced gastrointestinal muscle contractions were mediated by Ca^{2+} influx through voltage-sensitive channels [5, 9]. In smooth muscle from animal respiratory tract, the necessity of extracellular Ca^{2+} to maintain the response to vanadate and the lack of effect of verapamil on vanadate-induced contractions were reported [13, 31, 32], whereas in human bronchial smooth muscle it was found that vanadate only utilizes an intracellular source of Ca^{2+} that is sensitive to ryanodine [12]. However, our results indicate that ryanodine-sensitive receptors are not involved in vanadate-induced gallbladder contraction at low or high concentrations of vanadate, although high concentrations of vanadate probably induce Ca^{2+} release from intracellular stores. The controversial results found may reflect either different sources of Ca^{2+} depending on the species or tissues studied or different mechanisms responsible for the increase in $[Ca^{2+}]_i$. Cortijo *et al.* [12] suggested that the increase in $[Ca^{2+}]_i$ is due to the inhibition of Ca^{2+}-ATPase, but we would argue that it is the activation of the phosphotyrosine kinase pathway that is responsible for this increase.

Role of Membrane Transport Mechanisms in the Contractile Action of Vanadate

The plasmalemma of gallbladder smooth muscle cells, like other smooth muscle cells, contains several ion transport systems such as Na^+/K^+-ATPase and Ca^{2+}-ATPase. Inhibition of the Na^+/K^+ pump has been shown to cause contraction of smooth muscle [33]. Our observations that ouabain, a known blocker of Na^+/K^+-ATPase, contracts gallbladder strips supports this idea. Vanadate has been proposed as an inhibitor of Na^+/K^+-ATPase in a number of tissues [10], although our results suggest that vanadate is unlikely to act to inhibit this enzyme in the gallbladder smooth muscle. Re-addition of K^+ after incubation of the strips in K^+-free medium induces an ouabain-sensitive relaxation that can be attributable to the reactivation of Na^+/K^+-ATPase [12, 33]. In our study, ouabain, but not vanadate, blocked K^+-induced relaxation, which is an indication that vanadate does not inhibit the pump. These results are in agreement with those reported by Cortijo *et al.* [12] in airway smooth muscle.

Na^+/Ca^{2+} exchange acts as one of the mechanisms of Ca^{2+} extrusion in many types of cells including cardiac and smooth muscle under physiological conditions [34, 35]. It has been shown that the Na^+/Ca^{2+} exchange system can move calcium in either direction across the cell membrane in exchange for sodium and that the direction is dependent on the prevailing electrochemical gradient for Na^+ [36–40]. Accordingly, a rise in intracellular Na^+ or a decrease in extracellular Na^+ is thought to augment $[Ca^{2+}]_i$ by inhibiting the normal Ca^{2+} extrusion or by increasing Ca^{2+} entry into the cells through a reverse-mode activity of the Na^+/Ca^{2+} exchanger [41–44]. This could explain the contraction that we found after exposing the gallbladder strips to a Na^+-deficient K–HS. The present findings that tissue exposure to Na^+-deficient K–HS does not affect vanadate-induced contraction suggests that this mechanism is not activated by vanadate in gallbladder smooth muscle.

Vanadate and phosphate compete in numerous biological systems, and it has been reported that inorganic phosphate inhibited airway smooth muscle response to vanadate [13]. However, our study demonstrates that vanadate does not compete with phosphate in ATP synthesis and that it is necessary to maintain the integrity of oxidative phosphorylation for vanadate-induced contraction, what is corroborated by the inhibition observed in the vanadate contractile response in the presence of the mitochondrial uncoupler CCCP.

Intracellular Pathways Stimulated by Vanadate

The action of vanadate on gallbladder muscle depends on stimulation of the cyclooxygenase (COX) pathway and thus is similar to the reported action of EGF [4]. It has recently been reported that vanadate increases COX activity by posttranslational phosphorylation of the COX-2 isoform in cerebral endothelial cells [45]. This fact may explain the reduction described by us in the vanadate-induced response in the presence of the COX inhibitor indomethacin. Phospholipase A_2 activity may also be regulated posttranslationally by direct changes in tyrosine enzyme phosphorylation [46], so that in the presence of vanadate an increase in the activity of this enzyme could be expected. The reduction in vanadate-induced contraction in the presence of the PLA_2 blocker mepacrine supports this hypothesis. Therefore, our study demonstrates for the first time a relationship between cytosolic tyrosine phosphorylation and arachidonic acid metabolism in the control of smooth muscle contraction.

As under our experimental conditions the diacylglycerol lipase inhibitor did not reduce the actions of vanadate, an increase in AA formation from the PLC-dependent pathway can be excluded. These results are supported by the lack of effects reported by us for the PLC inhibitors and the PKC inhibitor.

In summary, we conclude that vanadate exerts direct effects on gallbladder smooth muscle that are mainly mediated by protein tyrosine phosphorylation (see Fig. 9 for main conclusions). Our data support the hypothesis that protein tyrosine phosphorylation causes an increase in calcium influx through voltage-dependent channels and/or calcium release from intracellular stores, and reveal a new link between tyrosine phosphorylation and prostanoid production in smooth muscle gallbladder contraction.

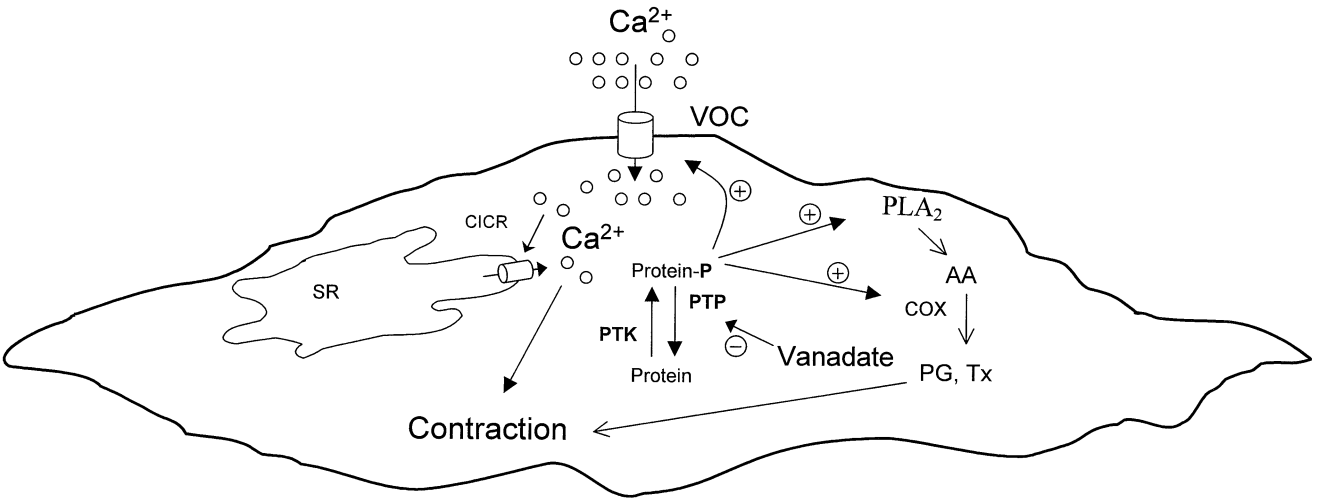

FIG. 9. A schematic model showing the proposed cellular mechanism of vanadate action in gallbladder smooth muscle contraction. Vanadate possibly inhibits protein tyrosine phosphatases (PTP), thus inducing an increase in protein tyrosine phosphorylation. This event might increase the conductance of voltage-operated channels (VOC) in the sarcolemma and permit influx of extracellular Ca^{2+}. Such an influx could also promote release of intracellular Ca^{2+} through calcium-induced calcium release (CICR), leading both mechanisms to contraction. Moreover, protein tyrosine phosphorylation would activate PLA_2 and cyclooxygenase (COX) activities, with a concomitant increase in arachidonic acid metabolism, which would also mediate gallbladder contraction. PG, prostaglandins; PTK: protein tyrosine kinase; Protein-P, protein phosphorylated in tyrosine residues; SR, sarcoplasmic reticulum; Tx, thromboxanes.

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References

1. Russell M and Hoeffler JP, Signal transduction and the regulation of cell growth. *J Invest Dermatol Symp Proc* **1:** 119–122, 1996.
2. Di Salvo J, Nelson SR and Kaplan N, Protein tyrosine phosphorylation in smooth muscle: A potential coupling mechanism between receptor activation and intracellular calcium. *Proc Soc Exp Biol Med* **214:** 285–301, 1997.
3. Berk BC, Alexander RW, Brock TA, Gimbrone MA Jr and Webb RC Vasoconstriction: A new activity for platelet-derived growth factor. *Science* **232:** 87–90, 1986.
4. Hollenberg MD, Tyrosine kinase pathways and regulation of smooth muscle contractility. *Trends Pharmacol Sci* **15:** 108–114, 1994.
5. Candura SM, Manzo L, Marraccini P, Coccini T and Tonini M, Investigation into vanadate-induced potentiation of smooth muscle contractility in the rabbit isolated ileum. *Life Sci* **54:** 237–244, 1994.
6. Di Salvo J, Steusloff A, Semenchuk L, Satoh S, Kolquist K and Pfitzer G, Tyrosine kinase inhibitors suppress agonist-induced contraction in smooth muscle. *Biochem Biophys Res Commun* **190:** 968–974, 1993.
7. Di Salvo J, Pfitzer G and Semenchuk LA, Protein tyrosine phosphorylation cellular Ca^{2+} and Ca^{2+} sensitivity for contraction of smooth muscle. *Can J Physiol Pharmacol* **72:** 1434–1439, 1994.
8. Swarup G, Cohen S and Garbers DL, Inhibition of phosphotyrosyl protein phosphatase activity by vanadate. *Biochem Biophys Res Commun* **107:** 1104–1109, 1982.
9. Laniyonu A, Saifeddine M, Ahmad S and Hollenberg MD, Regulation of vascular and gastric smooth muscle contractility by pervanadate. *Br J Pharmacol* **113:** 403–410, 1994.
10. Nechay BR, Mechanisms of action of vanadium. *Annu Rev Pharmacol Toxicol* **24:** 501–524, 1984.
11. Zhou Q, Satake N and Shibata S, The contractile mechanism of sodium metavanadate in isolated rat aortae. *J Cardiovasc Pharmacol* **30:** 84–89, 1997.
12. Cortijo J, Villagrasa V, Marti-Cabrera M, Villar V, Moreau J, Advenier CH, Morcillo EJ and Small RC, The spasmogenic effects of vanadate in human isolated bronchus. *Br J Pharmacol* **21:** 1339–1349, 1997.
13. Nayler RA and Sparrow MP, Mechanism of vanadate-induced contraction of airways smooth muscle of the guinea-pig. *Br J Pharmacol* **80:** 163–172, 1983.
14. Hudgins PM and Bond GH, Alteration by vanadate of contractility in vascular and intestinal smooth muscle preparations. *Pharmacology* **23:** 156–164, 1981.
15. Garcia AG, Jurkiewicz A and Jurkiewicz NH, Contractile effects of vanadate and other vanadate compounds on the rat vas deferens. *Eur J Pharmacol* **70:** 17–23, 1981.
16. Tallarida RJ and Murray RB, *Manual of Pharmacologic Calculations with Computer Programs*. Springer-Verlag, New York, 1991.
17. Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe SI, Itoh N, Shibaya M and Fukami Y, Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* **262:** 5592–5595, 1987.
18. Gazit AP, Yaish C, Gilon C and Levitzki A, Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. *J Med Chem* **32:** 2344–2352, 1989.
19. Lee KY, Biancani P and Behar J, Calcium sources utilized by cholecystokinin and acetylcholine in the cat gallbladder muscle. *Am J Physiol* **256:** G785–G788, 1989.
20. Somlyo AV and Somlyo AP, Strontium accumulation by sarcoplasmic reticulum and mitochondria in vascular smooth muscle. *Science* **174:** 955–958, 1971.
21. Yasuda N and Sakai Y, A possible explanation for effects of

Sr^{2+} on contraction–relaxation cycle in canine stomach. *Comp Biochem Physiol A* **78A:** 35–41, 1984.
22. Ito K, Takakura S, Sato K and Sutko JL, Ryanodine inhibits the release of calcium from intracellular stores in guinea pig aortic smooth muscle. *Circ Res* **58:** 730–734, 1986.
23. Muramatsu I, Itoh H, Lederis K and Hollenberg MD, Distinctive actions of epidermal growth factor-urogastrone in isolated smooth muscle preparations from guinea pig stomach: Differential inhibition by indomethacin. *J Pharmacol Exp Ther* **245:** 625–631, 1988.
24. Chuang M and Severson DL, Inhibition of diacylglycerol metabolism in isolated cardiac myocytes by U-57908 (RHC 80267), a diacylglycerol lipase inhibitor. *J Mol Cell Cardiol* **22:** 1009–1016, 1990.
25. Sakuta H and Yoneda I, Inhibition by SKF 525A and quinacrine of endogenous glibenclamide-sensitive K^+ channels in follicle-enclosed *Xenopus* oocytes. *Eur J Pharmacol* **252:** 117–121, 1994.
26. Filipeanu CM, Brailoiu E, Huhurez G, Slatineanu S, Baltatu O and Branisteanu DD, Multiple effects of tyrosine kinase inhibitors on vascular smooth muscle contraction. *Eur J Pharmacol* **281:** 29–35, 1995.
27. Feng GS, Hui CC and Pawson T, SH_2-containing phosphotyrosine phosphatase as a target of protein tyrosine kinases. *Science* **259:** 1607–1611, 1993.
28. Vogel W, Lammers R, Huang J and Ullrich A, Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science* **259:** 1611–1614, 1993.
29. Kaplan N and Di Salvo J, Coupling between [arginine^8]-vasopressin-activated increases in protein tyrosine phosphorylation and cellular calcium in A7r5 aortic smooth muscle cells. *Arch Biochem Biophys* **326:** 271–280, 1996.
30. Gould EM, Rembold CM and Murphy RA, Genistein, a tyrosine kinase inhibitor, reduces Ca^{2+} mobilization in swine carotid media. *Am J Physiol* **268:** C1425–C1429, 1995.
31. Cortijo J, Santamaria P, Anton M, Villagrasa V and Morcillo EJ, Effects of vanadate on responses of guinea-pig isolated trachea to spasmogens. *J Pharm Pharmacol* **45:** 54–58, 1993.
32. Lee SH, Hwang TT and Jung JS, Differential actions of AlF_4^- and vanadate on canine trachealis muscle. *Pflügers Arch* **427:** 295–300, 1994.
33. Chideckel EW, Frost JL, Mike P and Fedan JS, The effect of ouabain on tension in isolated respiratory tract smooth muscle of humans and other species. *Br J Pharmacol* **92:** 609–614, 1987.
34. Blaustein MP, Sodium–calcium exchange in mammalian smooth muscles. In: *Sodium–Calcium Exchange* (Eds. Allen TJ, Noble D and Reuter H), pp. 208–232. University Press, Oxford, 1989.
35. Morel N and Godfraind T, Sodium/calcium exchange in smooth-muscle microsomal fractions. *Biochem J* **218:** 421–427, 1984.
36. Carafoli E, How calcium crosses plasma membranes including the sarcolemma. In: *Calcium Antagonist and Cardiovascular Disease* (Ed. Opie LH), pp. 29–42. Raven Press, New York, 1984.
37. Schatzmann HJ, Calcium extrusion across the plasma membrane by the calcium pump and the Ca^{2+}/Na^+ exchange system. In: *Calcium and Cell Physiology* (Ed. Marme D), pp. 18–52, Springer-Verlag, Berlin, 1985.
38. Sheu SS and Blaustein MP, Sodium/calcium exchange and regulation of cell calcium and contractility in cardiac muscle with a note about vascular smooth muscle. In: *Heart and Cardiovascular System* (Eds. Fozzard HA, Haber E, Jennings RB, Katz AM and Morgan HE), pp. 509–536. Raven Press, New York, 1986.
39. Mechmann S and Pott L, Identification of Na-Ca exchange current in single cardiac myocytes. *Nature* **319:** 597–599, 1986.
40. Kimura J, Noma A and Irisawa H, Na-Ca exchange current in mammalian heart cells. *Nature* **319:** 596–597, 1986.
41. Brading AR, Burnett M and Sneddon P, The effect of sodium removal on the contractile responses of the guinea-pig taenia coli to carbachol. *J Physiol* (Lond) **306:** 411–429, 1980.
42. Lee CO, 200 years of digitalis: The emerging central role of the sodium ion in the control of cardiac force. *Am J Physiol* **249:** C367–C378, 1985.
43. Ohata H, Kawanishi T, Kawanishi M, Uneyama C, Takahashi M and Momose K, Spontaneous oscillations of cytoplasmic free calcium ion concentration in cultured smooth muscle cells from guinea pig ileum. *Jpn J Pharmacol* **63:** 83–91, 1993.
44. Nadal A, Valdeolmillos M and Soria B, Metabolic regulation of intracellular calcium concentration in mouse pancreatic islets of Langerhans. *Am J Physiol* **267:** E769–E774, 1994.
45. Parfenova H, Balabanova L and Leffler CW, Posttranslational regulation of cyclooxygenase by tyrosine phosphorylation in cerebral endothelial cells. *Am J Physiol* **274:** C72–C81, 1998.
46. Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A and Davis RJ, $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell* **72:** 269–278, 1993.