[Ca²⁺]_i-sensitive, IP₃-independent Ca²⁺ influx in smooth muscle of rat vas deferens revealed by procaine

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1 The actions of procaine (10 mM) on noradrenaline-induced effects on ⁴⁵Ca-influx, ⁴⁵Ca-efflux, ⁴⁵Ca-content, total inositol phosphates, inositol-1,4,5-trisphosphate, and contractile status of the rat vas deferens were examined.

2 Noradrenaline alone had no effect on ⁴⁵Ca-influx or ⁴⁵Ca-content, but released Ca²⁺ from intracellular stores as indicated by an increased ⁴⁵Ca-efflux and increased total inositol phosphates, specifically inositol-1,4,5-trisphosphate, leading to contraction of the rat vas deferens.

3 Noradrenaline, in the presence of 10 mM procaine, increased ⁴⁵Ca-influx and ⁴⁵Ca-content. Procaine blocked the noradrenaline-induced ⁴⁵Ca-efflux, the increase in total inositol phosphates, the increase in inositol-1,4,5-trisphosphate, and contraction.

4 The noradrenaline-induced increase in ⁴⁵Ca influx which was observed in the presence of procaine was abolished by phentolamine and nifedipine but was not altered significantly by propranolol suggesting that, in the presence of procaine, noradrenaline activates dihydropyridine-sensitive calcium channels through α -adrenoceptors.

5 These findings indicate that, in the rat vas deferens, noradrenaline induces contraction by releasing intracellularly stored Ca²⁺. The effects of procaine appear to be due to its ability to block the release of Ca²⁺ from intracellular stores. Furthermore, the simultaneous increase in ⁴⁵Ca influx and inhibition of inositol-1,4,5-trisphosphate formation in tissues treated with procaine plus noradrenaline indicates that Ca²⁺ influx is independent of inositol-1,4,5-trisphosphate formation.

Keywords: Smooth muscle; vas deferens; inositol trisphosphate; IP₃; calcium influx; calcium efflux; procaine

Introduction

In the rat vas deferens depolarizing concentrations of K⁺ increase Ca²⁺ influx and exchangeable Ca²⁺ content (Hay & Wadsworth, 1984; Khoyi *et al.*, 1988). In contrast, noradrenaline (NA) does not stimulate Ca²⁺ influx but increases its efflux into both normal physiological salt solution and Ca²⁺-free medium, resulting in decreased Ca²⁺ content of the muscle (Khoyi *et al.*, 1988). These results suggest that NA-induced contractions in the rat vas deferens primarily involve intracellular calcium. An apparent inconsistency with this hypothesis is the observation that contractions of vas deferens induced by NA are inhibited by the organic Ca²⁺ channel blocker, nifedipine (Feddersen *et al.*, 1988; Vesperinas *et al.*, 1989; personal observations). One possible explanation for this paradoxical observation is that NA initially produces a nifedipine-sensitive Ca²⁺ influx, which then causes a secondary release of Ca²⁺ from intracellular stores via a Ca²⁺-induced Ca²⁺ release mechanism. The subsequent increase in intracellular Ca²⁺ might inactivate ⁴⁵Ca influx in a manner similar to the inactivation of potential-dependent calcium currents by intracellular Ca²⁺ (Hagiwara & Byerly, 1981; Tsien, 1983; Chad *et al.*, 1984; Pacaud *et al.*, 1987) and help to explain previous observations that NA did not stimulate Ca²⁺ influx (Khoyi *et al.*, 1988).

If this hypothesis is correct, agents capable of preventing the release of Ca²⁺ from the sarcoplasmic reticulum should prevent the inactivation of ⁴⁵Ca influx resulting in an increase in ⁴⁵Ca influx upon application of NA. Procaine, at a concentration (10 mM) which is known to prevent the release of Ca²⁺ from the sarcoplasmic reticulum (Itoh *et al.*, 1981; 1982; Imai *et al.*, 1984; Ahn & Karaki, 1988) would be expected to have this effect. Therefore, the effects of procaine on ⁴⁵Ca fluxes, generation of inositol phosphates and the

development of tension were examined in the rat vas deferens.

Methods

Male albino rats, weighing 250–350 g, were killed by exsanguination following CO₂ anaesthesia. Vasa deferentia were removed and cleaned of vascular and connective tissue. The luminal contents of the vasa were removed by flushing with physiological salt solution (PSS). For some studies, animals were injected with reserpine 48 h (5 mg kg⁻¹) and 24 h (2.5 mg kg⁻¹) before each experiment to deplete the vas deferens of endogenous NA and reduce the potential confounding effects related to release of endogenous NA by agents such as procaine (see below). For similar reasons some studies were conducted with vasa deferentia which were sympathetically denervated by a modification of the 6-hydroxydopamine (6-OHDA) method of Aprigliano & Hermsmeyer (1976). Briefly, tissues were incubated with 6-OHDA (250 µg ml⁻¹) and glutathione (20 µM) in buffer-free PSS (pH 4.9) for two 15 min periods separated with an interval of 10 min in normal PSS (Khoyi *et al.*, 1988). Tissues were then washed thoroughly and kept at room temperature overnight in oxygenated PSS at pH 7.4. The next day the vasa deferentia were warmed to 37°C in oxygenated PSS and used in one of the experimental protocols as described below. A lack of contraction in response to electrical field stimulation (EFS), using parameters known to excite sympathetic nerves in this tissue was accepted as evidence of denervation.

Contraction studies

For contraction studies, tissues were mounted in isolated organ chambers and isometric contractions were recorded by

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conventional methods that have been described previously (Khoyi *et al.*, 1987). Krebs buffer was the PSS used in these studies and was of the following composition (mM): NaCl 110, KCl 4.6, CaCl_2 2.5, KH_2PO_4 1.15, MgSO_4 1.15, NaHCO_3 24.5, glucose 5.5. Tissues were equilibrated for 60 min at a resting tension of 1 g prior to challenge with NA (10 μM). Tissues were then washed several times and allowed to re-equilibrate in drug-free PSS for 15 min before a 15 min incubation period with procaine (10 mM). The tissues were then rechallenged with NA. For the studies which utilized Ca^{2+} -free PSS the CaCl_2 was omitted and EGTA (50 μM) was included. In these experiments the control response to NA (10 μM) was obtained after a 15 min incubation in Ca^{2+} -free medium.

Subsequently, Ca^{2+} -containing medium was introduced for 15 min (to reload intracellular stores). Next the tissues were re-incubated in Ca^{2+} -free medium plus procaine (10 mM) for 15 min before the tissue was rechallenged with NA.

Measurement of NA overflow

Tissues were incubated in an organ chamber and PSS solution was changed every 3 min. Samples of the PSS solution (3.5 ml) were transferred every 3 min to ice-cold tubes containing 0.7 ml of a solution containing 0.4 M perchloric acid, 1.3 mM Na_2EDTA and 5.3 mM $\text{Na}_2\text{S}_2\text{O}_5$ to stabilize catecholamines. The samples were then extracted over alumina to concentrate catecholamines and the NA content measured by high performance liquid chromatography (h.p.l.c.) with electrochemical detection as described previously (Shinozuka *et al.*, 1988). The effect of drugs on the overflow of NA from the tissue was monitored in this manner.

Extraction, separation and measurement of inositol phosphates

This was done according to the method of Zhang & Buxton (1991). Briefly, prior to experiments, tissues were incubated in an oxygenated labelling buffer containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 0.6, NaH_2PO_4 0.6, MgCl_2 1.2, glucose 5, CaCl_2 0.5, HEPES 10, pH = 7.4 and NaHCO_3 5 along with [^3H]-*myo*-inositol (200 $\mu\text{Ci ml}^{-1}$) and LiCl (10 mM) for 3 h at 35°C. After 2 min exposure to NA (10 μM) in the presence or absence of procaine (10 mM for 15 min), tissues were homogenized in $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{HCl}$ (66:33:1, v/v), clarified by centrifugation at 1 000 g for 10 min at room temperature and the aqueous phase lyophilized to dryness. The pellet was resuspended in 500 μl of H_2O and filtered (0.45 μm nylon) for h.p.l.c. analysis. Separation of inositol phosphates was accomplished using a gradient chromatographic system consisting of H_2O and 2 M $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 3.8) run over a strong anion exchange column (Whatman Partisil 5-SAX, 10 cm) at a flow rate of 0.8 ml min^{-1} (100% H_2O to 100% 2 M $\text{NH}_4\text{H}_2\text{PO}_4$ over 30 min). Radioactivity was monitored continuously with a Radiomatic flow detector at 12–15% efficiency for ^3H . Inositol phosphate standards were well resolved and used to identify unknowns. Data were determined as area under the h.p.l.c. peak in counts per min (c.p.m.) for inositol-1,4,5-trisphosphate (IP_3) and each of the inositol phosphate isomers. For total inositol phosphates, c.p.m. from individual peaks were added together.

Measurement of ^{45}Ca influx

Influx of ^{45}Ca was measured by the method of Meisheri *et al.* (1981) as described previously (Khoyi *et al.*, 1988). Briefly, a PSS of the following composition (in mM) was used: NaCl 140, KCl 5, MgCl_2 1, CaCl_2 1.5, HEPES 5 and glucose 10. The PSS was maintained at pH 7.4 and gassed with 100% O_2 . After 90 min of equilibration, the tissues were exposed to PSS containing ^{45}Ca (0.6–0.8 $\mu\text{Ci ml}^{-1}$) and the drugs being studied. For experiments in which the effects of procaine were examined, this agent was added to the bathing solution

during the last 20 min of the equilibration period. For determination of ^{45}Ca influx, the exposure of ^{45}Ca was limited to 2 min. After incubation in ^{45}Ca -labelling solution, the tissues were immediately transferred to 10 ml of ice-cold (0.5°C) Ca^{2+} -free oxygenated PSS containing 2 mM EGTA (pH 7.2) for 2 min. All tissues were then immersed in 500 ml of this same solution for 48 min to remove extracellular ^{45}Ca . It has been shown that even during the first minutes of washing some of the intracellular ^{45}Ca is lost to the washing solution (Aaronson, 1989). However, several investigators have observed that the remaining intracellular ^{45}Ca after washout is proportional to the amount of intracellular ^{45}Ca present at the end of the labelling period (see Loutzenhiser *et al.*, 1985). The duration of the wash period in Ca^{2+} -free, ice-cold solution was determined by preliminary desaturation experiments. These experiments showed that the tissues lost approximately 25% of intracellular ^{45}Ca in the 50 min wash period. The tissues were then placed on filter paper and the vasa deferentia bisected into epididymal and prostatic segments. Each segment was then cut into small pieces (2–3 mm), blotted, and put in preweighed vials to determine wet weight. Three ml of EDTA solution (5 mM) was added to each vial which was then left overnight at room temperature. Finally, 3.5 ml scintillation cocktail (ScintiVerse I) was added to each vial and radioactivity was determined in a Beckman liquid scintillation counter. Tissue counts were converted to tissue Ca^{2+} content by using the specific activity of ^{45}Ca in the incubation medium, and influx was expressed as micromol $^{45}\text{Ca kg}^{-1}$ wet tissue weight.

Measurement of ^{45}Ca content

Tissues were equilibrated in PSS at 37°C for 90 min and then exposed to ^{45}Ca containing PSS for an additional 2 h to label the exchangeable intracellular Ca^{2+} pool. The tissues were then exposed to NA (10 μM) and procaine (10 mM) in the last 10 and 20 min of incubation, respectively. The ^{45}Ca content was then determined as described above.

Measurement of ^{45}Ca efflux

Tissues were equilibrated in PSS (37°C) and then exposed to ^{45}Ca containing medium for up to 3 h to achieve steady-state labelling of the exchangeable intracellular Ca^{2+} pool. Tissues were then passed at 2-min intervals through a series of test tubes that contained calcium-free medium to which EGTA (0.05 mM) had been added. Procaine (10 mM) was added to the experimental group at the 70th min of rinsing until the end of the experiment and NA (100 μM) was added to both groups during the 80th–90th min. At the end of each experiment, the radioactivity in each test tube and that of the tissue was determined as described above. The rate of ^{45}Ca efflux was then calculated as the fraction of radioactivity present in the tissue at the time of sampling. From this, an efflux curve was generated. The efflux data were obtained at 20°C rather than at 37°C to slow ^{45}Ca efflux and thereby assess more accurately the influence of NA on the rate of ^{45}Ca efflux.

Statistical analysis

All data are presented as means $\pm$ s.e. Student's *t* test was used for paired or unpaired data as appropriate. Analysis of variance was performed with Duncan's multiple range test for multiple comparisons between means. A $P < 0.05$ was considered significant.

Drugs used

Drugs used were phentolamine mesylate supplied by Ciba-Geigy, Summit, NJ, U.S.A., nifedipine supplied by Pfizer, New York, NY and noradrenaline (NA), propranolol, reserpine, 6-hydroxydopamine (6-OHDA), procaine and tetraethylammonium (TEA) supplied by Sigma Chemical, St. Louis, MO, U.S.A.

Results

Effect of procaine on the overflow of NA from rat vas deferens

Incubation of vas deferens with procaine (10 mM) caused a significant and maintained increase in the overflow of NA from the tissue for a period of 60 min (not shown). Therefore, unless otherwise stated, the experiments described below were carried out with denervated or reserpine-treated tissues. These procedures abolished the procaine-induced overflow of NA and eliminated complications due to release of endogenous NA.

Effects of procaine on the contractile response of rat vas deferens to NA

Figure 1 shows a representative example of the effect of procaine (10 mM) on the contractile response to NA (10 μ M) in normal PSS (2.5 mM Ca²⁺) and Ca²⁺-free PSS (0 mM Ca²⁺ + 50 μ M EGTA, see Methods section for protocol). In the presence of Ca²⁺, procaine (10 mM) reduced the contractile response of vasa deferentia to NA (10 μ M) such that after procaine only 2.8 $\pm$ 1.2% of the response remained ($P < 0.05$, compared to control, $n = 9$). Figure 1 also shows that the contraction to NA was markedly reduced in Ca²⁺-free PSS compared to that observed in normal PSS. The initial phasic component was reduced and the secondary maintained phase was abolished. Subsequent addition of procaine completely abolished the contractile response to NA ($n = 6$).

Effects of procaine on NA stimulated [³H]-inositol phosphate accumulation

Stimulation by NA produced a large increase in IP₃ accumulation (1.9 $\pm$ 0.05 fold, Figure 2) which was mirrored by similar increases in total inositol phosphates (2.26 $\pm$ 0.22 fold). Procaine (10 mM) at a concentration which blocked NA-induced contraction, abolished the effect of NA on inositol phosphates (Figure 2).

Figure 1 Representative examples of 6–9 experiments examining the effect of procaine on noradrenaline (NA)-induced contraction of rat vas deferens in Ca²⁺-containing Krebs solution (a) and in Ca²⁺-free solution (b, see Methods). The first trace in each panel shows the control contraction to NA (10 μ M) and the second trace shows the contraction to NA in the presence of procaine (10 mM for 15 min).

Figure 2 Effects of procaine (10 mM for 15 min) on accumulation of [³H]-inositol phosphates stimulated by noradrenaline (NA, 10 μ M for 2 min) in rat vas deferens. Pretreatment of the tissue with 10 mM procaine showed no effect on basal phosphoinositide metabolism (total IP from 4172 $\pm$ 1260 to 4183 $\pm$ 1335 c.p.m. mg⁻¹ tissue wet weight, IP₃ from 272 $\pm$ 107 to 300 $\pm$ 118 c.p.m. mg⁻¹ tissue wet weight). Open columns represent the effect of NA (10 μ M) in the absence of procaine and solid columns represent the levels in the presence of procaine as % of basal levels. Procaine completely blocked NA-induced generation of total inositol phosphates and IP₃. Values shown are mean $\pm$ s.d. of 3 experiments. Significant difference from control group: * $P < 0.05$; ** $P < 0.001$.

Effect of procaine and NA on ⁴⁵Ca influx

In reserpine-treated tissues, as previously shown (Khoyi *et al.*, 1988), NA had no effect on the rate of ⁴⁵Ca influx (Figure 3). However, when reserpine-treated tissues were incubated with procaine, exposure to NA significantly increased the rate of ⁴⁵Ca influx (Figure 3). This increased influx of ⁴⁵Ca was prevented by phentolamine but was not changed significantly by propranolol (data not shown). Procaine alone did not significantly increase ⁴⁵Ca influx in reserpine-treated tissues.

Figure 3 Effect of noradrenaline (NA, 10 μ M) and procaine (10 mM) on ⁴⁵Ca influx in vasa deferentia from reserpinized rats. The concentration of Ca²⁺ in the medium was 1.5 mM. Open columns represent basal levels of ⁴⁵Ca influx and hatched columns represent stimulation with NA. Note that NA significantly increased ⁴⁵Ca influx in the presence ($P < 0.05$, $n = 6$) but not in the absence ($n = 6$) of procaine.

Effect of nifedipine on ^{45}Ca influx produced by NA in the presence of procaine

Nifedipine blocked the increase in Ca^{2+} influx which is observed in tissues exposed to procaine plus NA. The combination of nifedipine and NA, without procaine, was no different from NA alone (Figure 4). In tissues that were not stimulated with NA, neither procaine, nifedipine nor a combination of the two had any significant effect on Ca^{2+} influx ($n = 4-8$, data not shown).

Effect of tetraethylammonium (TEA) on Ca^{2+} flux

TEA (20 mM) alone caused an increase in Ca^{2+} influx from $27.3 \pm 2.42 \mu\text{mol kg}^{-1}$ wet weight to $47.75 \pm 2.32 \mu\text{mol kg}^{-1}$ wet weight ($P < 0.05$, $n = 5$). In the presence of TEA, NA significantly reduced Ca^{2+} influx to $40.34 \pm 1.21 \mu\text{mol kg}^{-1}$ wet weight ($P < 0.025$, $n = 5$).

Effect of procaine on the ^{45}Ca content of vas deferens

The exchangeable ^{45}Ca content of vasa deferentia was measured after a 2 h incubation in PSS with ^{45}Ca . For this purpose, the rats were pretreated with reserpine and phenolamine ($1 \mu\text{M}$) was added to the incubation medium when tissues were not to be exposed to exogenous NA. Procaine (10 mM) and NA ($10 \mu\text{M}$), when present, were added during the last 20 and 10 min of the ^{45}Ca labelling period, respectively. The ^{45}Ca content of these tissues was increased moderately by procaine. The ^{45}Ca content of vasa deferentia was significantly increased by NA in the presence of procaine (Figure 5).

Effects of procaine on ^{45}Ca efflux

For ^{45}Ca efflux experiments, vasa deferentia from reserpine pretreated rats were utilized. After equilibration and incubation with ^{45}Ca containing medium, vasa deferentia were rinsed with Ca^{2+} -free medium in the absence or presence of 10 mM procaine for the entire period of the ^{45}Ca efflux experiment and NA ($100 \mu\text{M}$) was added for 10 min during the 80th–90th min of rinsing. Procaine did not change the rate of ^{45}Ca efflux in unstimulated tissues but completely abolished the stimulatory effect of NA on ^{45}Ca efflux (Figure 6).

Figure 4 Effect of nifedipine on ^{45}Ca influx induced by noradrenaline (NA) in the presence of procaine. Columns indicate tissues stimulated with $10 \mu\text{M}$ NA (column a), $10 \mu\text{M}$ NA + 10 mM procaine (column b), $10 \mu\text{M}$ NA + $1 \mu\text{M}$ nifedipine (column c) and $10 \mu\text{M}$ NA + 10 mM procaine + $1 \mu\text{M}$ nifedipine (column d). Note that nifedipine completely blocked the increase in ^{45}Ca influx induced by NA + procaine. The combination of nifedipine plus NA, without procaine, was no different from NA alone ($n = 4-7$).

Figure 5 ^{45}Ca -content of rat vasa deferentia under unstimulated conditions (column a), and in tissues exposed to noradrenaline ($10 \mu\text{M}$, column b), procaine (10 mM , column c) and the combination of NA plus procaine (column d). Note that the ^{45}Ca content of the vasa deferentia was increased by NA in the presence ($P < 0.05$), but not in the absence of procaine. Each column is the mean $\pm$ s.e.mean of 6–9 experiments.

Figure 6 Effect of procaine (10 mM) on ^{45}Ca efflux induced by noradrenaline (NA, $100 \mu\text{M}$) from vasa deferentia of reserpine pretreated rats. In tissues preincubated with procaine ($\blacktriangle$) the NA-induced increase in ^{45}Ca efflux which was seen in control tissues (Δ) was blocked ($n = 6$).

Discussion and conclusions

The results of the present study suggest that; (i) NA-induced contraction of the rat vas deferens is dependent on Ca^{2+} release from the sarcoplasmic reticulum and not on Ca^{2+} influx, because block of release of Ca^{2+} from sarcoplasmic reticulum by procaine results in inhibition of contraction; (ii) NA-induced Ca^{2+} influx is only apparent when IP_3 generation and release of Ca^{2+} from the sarcoplasmic reticulum are blocked by procaine, suggesting that in the absence of procaine, elevated cytosolic Ca^{2+} concentrations cause inactivation of Ca^{2+} influx; (iii) IP_3 generation is not necessary for the occurrence of Ca^{2+} influx during exposure of the tissue to procaine plus NA, because this combination blocked the generation of IP_3 but increased Ca^{2+} influx.

In the present study, NA contracted the vas deferens but increased neither ^{45}Ca influx nor ^{45}Ca content. However, it caused release of Ca^{2+} from intracellular stores, increased ^{45}Ca efflux and increased IP_3 generation. These findings are in agreement with other data (Khoi *et al.*, 1988) which indicate

(i) that in the vas deferens, as in dog coronary artery (Imai *et al.*, 1984), NA utilizes intracellularly stored Ca²⁺ for contraction, and (ii) that Ca²⁺ entry through the cell membrane seems to be unnecessary to provide free Ca²⁺ to activate the contractile protein. After exposure of the vas deferens to NA, either IP₃, or Ca²⁺-induced Ca²⁺ release from the sarcoplasmic reticulum, or both, may increase the intracellular Ca²⁺ concentration to a level which activates the contractile apparatus. However, a paradox exists in that while NA appears to translocate ⁴⁵Ca only from intracellular stores within the vas deferens, contractions induced by NA have been shown to be inhibited by nifedipine (Feddersen *et al.*, 1988; Vesperinas *et al.*, 1989). This organic calcium channel antagonist blocks voltage-sensitive Ca²⁺ channels without affecting Ca²⁺ gradient between intra- and extra-cellular mediums. It is possible that the method used in this study is not sensitive enough to detect a nifedipine-sensitive Ca²⁺ influx which may then act as a trigger for Ca²⁺-induced Ca²⁺ release from the sarcoplasmic reticulum, as has been suggested previously (Itoh *et al.*, 1986). It is also possible that nifedipine inhibits the contraction of vas deferens to NA through mechanisms other than the blockade of calcium channels (Rinaldi *et al.*, 1987; Jaquemond & Rougier, 1988; Xuan *et al.*, 1989). However, the present observation that NA increases ⁴⁵Ca influx in vasa deferentia that were preincubated with procaine and that this increased Ca²⁺ influx was blocked by nifedipine indicates that NA is capable of activating Ca²⁺ influx in this tissue through dihydropyridine-sensitive calcium channels.

Four possible explanations can be given to explain the absence of Ca²⁺ influx during stimulation with NA in the absence of procaine: (i) NA-induced increases in intracellular Ca²⁺ may indirectly inactivate Ca²⁺ influx by opening Ca²⁺-activated K⁺ channels. However, if this were the case, then blocking Ca²⁺-activated K⁺ channels should increase ⁴⁵Ca influx. In the present experiments, TEA, which blocks Ca²⁺-activated K⁺ channels in other smooth muscles (Carl *et al.*, 1993), did not increase NA induced ⁴⁵Ca influx, but in fact significantly decreased ⁴⁵Ca influx; (ii) the Ca²⁺ which enters the cell is immediately pumped out by Ca²⁺-ATPase. This also is unlikely because, even if there were no net change in intracellular Ca²⁺ content, one would expect that an exchange of the ⁴⁵Ca with the non-radioactive intracellular Ca²⁺ would result in an 'apparent' ⁴⁵Ca influx; (iii) NA may directly reduce Ca²⁺ channel activity in addition to Ca²⁺ inactivation of Ca²⁺ channels (Imaizumi *et al.*, 1991). This also is unlikely because NA increased ⁴⁵Ca influx in the presence of procaine. We favour the fourth possibility, which is: (iv) NA-induced increases in intracellular Ca²⁺ from the sarcoplasmic reticulum may directly inactivate the Ca²⁺ influx in a manner similar to the inactivation of potential-dependent calcium currents by intracellular Ca²⁺ (Hagiwara & Byerly, 1981; Tsien, 1983; Chad *et al.*, 1984; Pacaud *et al.*, 1987).

In the presence of procaine, NA was unable to contract the vas deferens despite an increased ⁴⁵Ca influx and increased ⁴⁵Ca content of the tissue. Furthermore, procaine completely inhibited the NA-induced ⁴⁵Ca efflux. This indicates that

procaine blocked the NA-induced release of Ca²⁺ from intracellular stores and that this is the primary source of Ca²⁺ for contraction in this tissue. In the presence of procaine, NA did not increase IP₃ generation but did increase ⁴⁵Ca influx. This increase of ⁴⁵Ca influx was mediated through α -adrenoceptor stimulation and occurred through dihydropyridine-sensitive calcium channels. The increase of ⁴⁵Ca influx in the absence of any increase in IP₃ formation is interesting because it has been suggested that in other cells, IP₃ mediates activation of receptor operated Ca²⁺ influx (see review by Putney, 1992). However, it is shown here that procaine inhibited NA-induced IP₃ formation without inhibiting Ca²⁺ influx. These results indicate that, for the rat vas deferens, IP₃ formation was not a required intermediate step in the NA-induced increase of Ca²⁺-influx.

The present observations that procaine prevented the NA-induced increase in ⁴⁵Ca efflux (Figure 6) and increased ⁴⁵Ca content (Figure 5) of the vas deferens, are in accord with the inhibitory effect of procaine on the release of Ca²⁺ from calcium storage sites (Endo, 1977; Itoh *et al.*, 1981; 1982). Furthermore, others have provided evidence in favour of the target for the action of procaine being on the sarcoplasmic reticulum in that procaine has no effect on the pCa-tension relationship in chemically skinned arterial smooth muscle cells (Itoh *et al.*, 1981; 1982). This implies that procaine affects neither the contractile mechanisms directly nor the ability of Ca²⁺ to activate it.

The methodology used in this study is dependent upon retention of Ca²⁺ which has entered the cell. Any Ca²⁺ which is not retained within the cell is not detected. Therefore, it cannot be determined whether NA causes inactivation of Ca²⁺ influx due to raising of cytoplasmic Ca²⁺ or prevents intracellular retention of Ca²⁺ in the sarcoplasmic reticulum. Electrophysiological analysis of the inactivation of the Ca²⁺ channels under different conditions may help to answer these questions.

It is well known that IP₃ will cause Ca²⁺ release from the sarcoplasmic reticulum (Berridge, 1984; Somlyo *et al.*, 1985). The present results are, to our knowledge, the first observation that procaine may inhibit indirectly intracellular Ca²⁺ release by blocking IP₃ formation, as well as directly inhibiting Ca²⁺ release from sarcoplasmic reticulum (Endo, 1977). The mechanism of procaine-induced inhibition of IP₃ formation cannot be determined from these experiments but may be associated with its well known membrane stabilizing activity.

In summary, these results provide further evidence that intracellularly stored Ca²⁺ is the primary mediator of NA-induced contraction in the rat vas deferens. The use of procaine shows that this intracellular Ca²⁺ also inactivates Ca²⁺ influx. The existence of NA-induced Ca²⁺ influx in the presence of procaine while IP₃ formation was blocked indicates that NA-induced Ca²⁺ influx in vas deferens smooth muscle is independent of IP₃ formation.

This work was supported by a grant from the American Heart Association (M.A.K.) and NIH Grants HL 38126 (D.P.W.), HD 26227 (I.L.O.B.).

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(Received November 23, 1992)

Revised July 26, 1993

Accepted August 3, 1993)