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Mobilization of Intracellular Calcium in Cultured Vascular Smooth Muscle Cells by Uridine Triphosphate and the Calcium Ionophore A23187

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Abstract. The known action of uridine triphosphate (UTP) to contract some types of vascular smooth muscle, and the present finding that it is more potent than adenosine triphosphate in eliciting an increase in cytosolic Ca^{2+} concentration in aortic smooth muscle, led us to investigate the mode of action of this nucleotide. With this aim, cultured bovine aorta cells were subjected to patch-clamp methodologies under various conditions. Nucleotide-induced variations in cytosolic Ca^{2+} were monitored by using single channel recordings of the high conductance Ca^{2+}-activated K^+ (Maxi-K) channel within on-cell patches as a reporter, and whole-cell currents were measured following perforation of the patch. In cells bathed in Na^+-saline, UTP (>30 nM) induced an inward current, and both Maxi-K channel activity and unitary current amplitude of the Maxi-K channel transiently increased. Repetitive exposures elicited similar responses when 5 to 10 min wash intervals were allowed between challenges of nucleotide. Oscillations in channel activity, but not oscillation in current amplitude were frequently observed with UTP levels > 0.1 μM. Cells bathed in K^+ saline (150 mM) were less sensitive to UTP (~5-fold), and did not show an increase in unitary Maxi-K current amplitude. Since the increase in amplitude occurs due to depolarization of the cell membrane, a change in amplitude was not observed in cells previously depolarized with K^+ saline. The enhancement of Maxi-K channel activity in the presence of UTP was not diminished by Ca^{2+} entry blockers or by removal of extracellular Ca^{2+}. However, in the latter case, repetitive responses progressively declined. These observations, as well as data comparing the action of low concentrations of Ca^{2+} ionophores (<5 μM) to that of UTP indicate that both agents elevate cytosolic Ca^{2+} by mobilization of this ion from intracellular pools. However, the Ca^{2+} ionophore did not cause membrane depolarization, and thus did not change unitary current amplitude. The effect of UTP on Maxi-K channel activity and current amplitude was blocked by pertussis toxin and by phorbol 12-myristate 13-acetate (PMA), but was not modified by okadaic acid, or by inhibitors of protein kinase C (PKC). Our data support a model in which a pyrimidinergic receptor is coupled to a G protein, and this interaction mediates release of Ca^{2+} from intracellular pools, presumably via the phosphatidyl inositol pathway. This also results in activation of membrane channels that give rise to an inward current and depolarization. Ultimately, smooth muscle contraction ensues. PKC does not appear to be directly involved, even though the UTP response is blocked by low nM levels of PMA. While the latter data implicate PKC in diminishing the UTP response, agents that inhibit either PKC or phosphatase activity did not prevent abolition of UTP responses by PMA, nor did they modify basal channel activity.

Key words: Maxi-K channel — Aortic smooth muscle — Pyrimidine nucleotides — UTP — Protein kinase C

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Introduction

Both UTP and adenosine triphosphate (ATP) elicit contractions of a variety of vascular smooth muscle preparations (von Kugelgen & Starke, 1990), and in some tissues evidence supports the presence of separate receptors for these nucleotides (von Kugelgen, Haussinger & Starke, 1987; von Kugelgen & Starke, 1990). UTP is particularly effective in human cerebral arteries where it elicits long-lasting tensions that are occasionally oscillatory (Urquilla, 1978). Information about pyrimidinergic receptors and their coupling to second messenger pathways is limited (*see* Seifert & Schultz, 1989). In cell types other than smooth muscle, UTP has been noted to affect a number of cellular functions such as cell aggregation, generation of inositol phosphates, Ca^{2+} influx, and release of Ca^{2+} from intracellular pools (*see* Seifert & Schultz, 1989). In some preparations, UTP-elicited responses are mediated via G proteins as demonstrated by inhibition of the responses following treatment with pertussis toxin (Seifert, Burde & Schultz, 1989; Seifert et al., 1989).

Considerably more data is available regarding the effects of ATP on smooth muscle (*see* Bean, 1992). In general, ATP depolarizes vascular smooth muscle cells either by activating a membrane pathway that is primarily Na^+ permeable (Suzuki, 1985), or by opening a receptor-operated, cation-selective channel (Benham & Tsien, 1987; Benham, 1989). Identification of this channel in the last cited study revealed a conductance of ~5 pS (110 mM Ca^{2+} saline) and a selectivity for Ca^{2+} over Na^+ of 3. This channel is believed to be directly activated by ATP and the resulting Ca^{2+} influx contributes towards activation of the contractile process. Additional evidence favoring direct activation of this channel by ATP, rather than by a second messenger, was obtained by measurements of the short latency of channel activation following exposure of guinea pig bladder smooth muscle cells to ATP (Inoue & Brading, 1990). However, in this preparation the Ca^{2+} current conveyed by the ATP-activated channel did not significantly affect contraction. In other smooth muscle preparations, the ATP-induced depolarization increases cytosolic Ca^{2+} by causing the activation of voltage-dependent Ca^{2+} channels (Friel, 1988). In these latter studies, an ATP-induced release of Ca^{2+} from intracellular pools as occurs in frog heart cells (Niedegerke & Page, 1981) was not ruled out. Also not considered was whether the nucleotide receptor is coupled to the Ca^{2+} channel via a G protein, as is the case for coupling of the muscarinic receptor to a K^+ channel in heart cells (Breitwieser & Szabo, 1985; Pfaffinger et al., 1985).

In this study, the effects of nucleotides on aortic smooth muscle cells were characterized using the high conductance Ca^{2+}-activated K^+ (Maxi-K) channel as a reporter. UTP was found to affect both the activity and unitary current amplitude of the Maxi-K channel. The oscillatory enhancement of Maxi-K channel activity within on-cell patches elicited by UTP as described here and in previous studies (*see* Martin, 1992) is believed to be a consequence of an IP_3-mediated Ca^{2+} release process. On the other hand, the nucleotide-induced monophasic variation of channel current amplitude reflects a phasic change of membrane potential. The basis for the oscillations in the level of cytosolic Ca^{2+} is not understood, but both positive and negative feedback processes are presumably operative. "Ca^{2+} releases Ca^{2+}" may be responsible for the positive feedback process. Proposals for negative feedback include activation of PKC, which in turn inhibits phospholipase C (PLC), or a decoupling of activated G proteins from the enzyme (Drummond, 1986; Smith, Uhing & Snyderman, 1987). Preliminary data regarding part of this work have appeared in abstract form (Sanchez et al., 1991*a*, *b*).

Materials and Methods

Cell Culture

The procedures routinely used for preparing smooth muscle cells from bovine thoracic aorta for culture and subsequent use in patch-clamp experiments have been described (Williams et al., 1988).

Solutions and Chemicals

The major ion constituents of the bathing and electrode solutions (in mM) are included in the figures along with the electrode-patch configurations. All solutions were buffered at pH 7.3 with 5 to 10 mM HEPES. In general, the bathing Na^+ saline contained (in mM) 135 or 145 Na^+, 5 K^+, 1 or 2 Ca^{2+} and 1 to 5 Mg^{2+}. The high K^+ saline used for bathing cells and filling electrodes contained 150 K^+, 1 or 2 Mg^{2+}, and 0 to 2 Ca^{2+}. All of the nucleotides used in this study were purchased from Sigma, as were the Ca^{2+} ionophores, ionomycin and A23187. Okadaic acid and BAPTA/AM were bought from CalBiochem. Pertussis toxin and activators/inhibitors of PKC, which included staurosporine, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride (H-7), D-sphingosine, and cremophor, were obtained from Sigma, as was inositol-1,4, 5-triphosphate.

Electrical Measurements

Single channel currents were monitored from both cell-attached and excised membrane patches (Hamill et al., 1981). The procedures used for preparing electrodes, superfusing cells in the experimental chamber, recording and analysis of channel currents

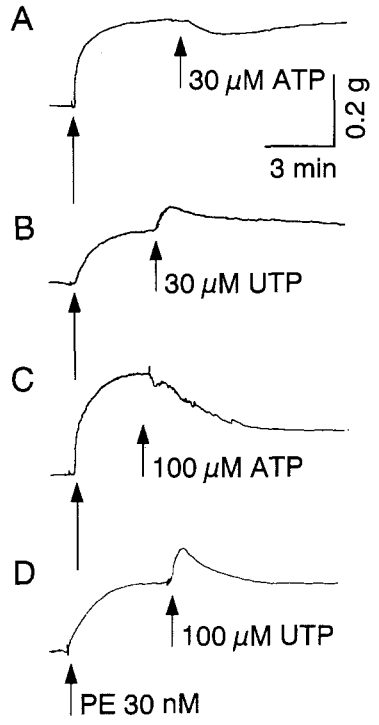

Fig. 1. Modification of tensions evoked by phenylephrine (PE) in rat aortic smooth muscle by UTP and ATP. After attaining a steady level of tension following exposure of the smooth muscle to 3×10^{-8} M PE, the preparations were exposed to either UTP (*B* and *D*) or ATP (*A* and *C*) at the concentrations noted.

were identical to those reported previously (Barros et al., 1986; Williams et al., 1988). One addition to the protocol for recording current was the use of current averaging. Since most patches contained more than one channel, percent open time was obtained by measuring the number of channels (n) and the magnitude of the unitary channel current (I_μ). Percent open time is $I_{average}/nI_\mu$. To determine n, we applied sufficient depolarization to activate all channels. In patches that contained five or fewer channels, this procedure provided a reliable measure of n. Perforation of on-cell patches for whole-cell recording was achieved by including 250 μg/ml nystatin (Sigma) in the pipette solution (Horn & Marty, 1988).

Results

Effects of Nucleotides on Smooth Muscle Contraction

Both ATP and UTP have pronounced effects on smooth muscle contractility. For example, as shown in Fig. 1, when rat aortic rings are treated with 3×10^{-8} M phenylephrine, a contraction ensues. Addition of 30 μM ATP to this preparation transiently reduces the contraction (Fig. 1*A*), while 30 μM UTP transiently increases contraction of the tissue (Fig. 1*B*). Higher concentrations of ATP (100 μM; Fig. 1*C*) caused a substantial and long-lasting reduction in the phenylephrine-induced tension. In contrast, 100 μM UTP elicited another transient contraction of this preparation. It is expected that the ATP-evoked relaxation of a smooth muscle preparation with an intact endothelium is mediated by purinoceptors which promote release of an endothelium-derived relaxant factor (Boeynaems & Pearson, 1990). Since UTP does not mimic the action of ATP, it must not activate the purinoceptor in rat aortic endothelial cells. Moreover, the UTP-induced contraction of rat aorta suggests that receptors may exist on smooth muscle cells which recognize pyrimidine nucleotides. To characterize the mode of action of UTP, we used the activity of the high conductance Ca^{2+}-activated K^+ (Maxi-K) channel to monitor changes of intracellular Ca^{2+} concentration during exposure of cultured bovine aortic smooth muscle cells to this nucleotide, or to Ca^{2+} ionophores.

Characteristics of UTP- and Ca^{2+} Ionophore-induced Maxi-K Channel Activation

In Fig. 2*A* and *C*, a transient enhancement of Maxi-K channel activity within on-cell patches occurred upon exposing one cell to 3.0 μM UTP (*A*), and another cell to 2.0 μM of the Ca^{2+} ionophore, A23187(*C*). The channel activation induced by UTP (as well as by other nucleotides—*see below*) was always transient, lasting 1 to 5 min regardless of whether the time course was monophasic or oscillatory. The transient increase in percent open time of the channels elicited by the Ca^{2+} ionophores A23187 and ionomycin was only observed at low ionophore concentrations. At values above *ca.* 5.0 μM for A23187, or *ca.* 0.5 μM for ionomycin, a component of channel activity was sustained. Oscillations in activity, which were frequently observed following application of UTP, were never seen in recordings from cells exposed to Ca^{2+} ionophores. Another characteristic of the Maxi-K channel response, which is limited to those elicited by nucleotides, was the transient increase in amplitude of the unitary channel current (Fig. 2*A*, lower plot). The time course of change in unitary current amplitude evoked by UTP is similar to that of the appearance of an inward current recorded under whole-cell voltage clamp following perforation of the on-cell patch (*see* Materials and Methods) and subsequent exposure to 3.0 μM UTP (Fig. 2*B*). The unitary current amplitude did not change during the period of increased channel activity evoked by exposing cells to Ca^{2+} ionophores (Fig. 2*C*, lower plot). An inward current

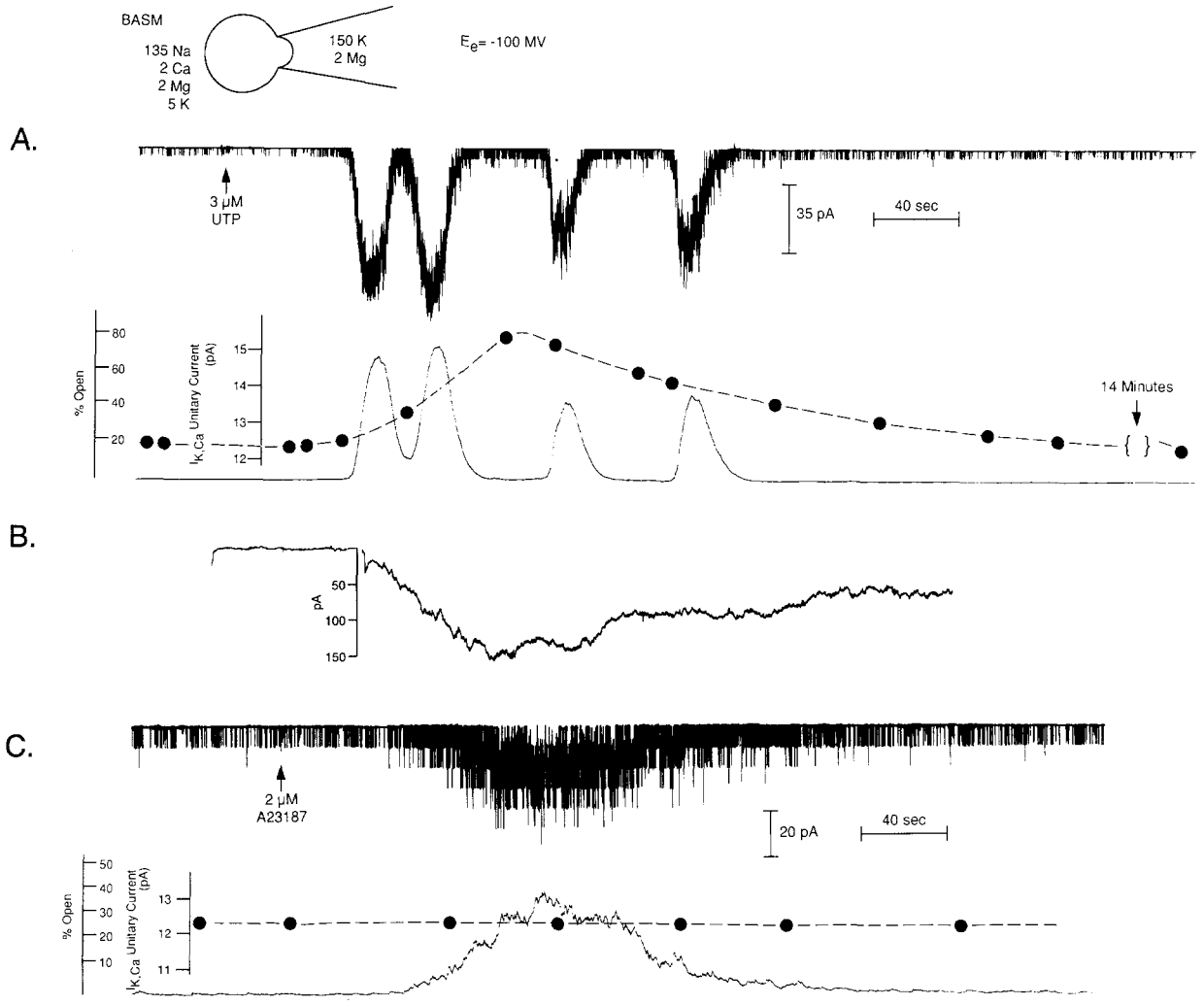

Fig. 2. Effect of UTP and Ca^{2+} ionophore on intracellular Ca^{2+} concentrations in bovine aortic smooth muscle. UTP, but not A23187 elicits an oscillatory increase in Maxi-K channel activity within on-cell patches of bovine aorta cells. In Na^+ saline, UTP (*A*, plot), but not A23187 (*C*, plot), also evoked a monophasic increase in amplitude of the unitary channel current. The time course of the change in unitary channel current amplitude is similar to that of an inward current measured under whole-cell voltage clamp (perforated patch) following exposure of cells to UTP (*B*).

was also not elicited in cells superfused with saline containing 2 to 5 μM A23187 (*data not shown*). Furthermore, neither an inward current, nor an increase in amplitude of Maxi-K channel current occurred in cells that were depolarized by being bathed in 150 mM K^+ saline following exposure to UTP (*see below*).

Several experiments were carried out with cells bathed in Na^+-free saline (N-methyl-D-glucamine substituting for Na^+) containing 5 mM K^+. The sensitivity to UTP declined at least fivefold (Fig. 3) and, in this respect, the concentration dependence was similar to that obtained in cells bathed in 150 mM K^+ saline. To elicit a response comparable to that recorded in Na^+ saline containing 3.0 μM UTP (Fig. 2*A*), we had to elevate the concentration of UTP to 30 μM (Fig. 3). In addition, in this situation UTP had a different effect on unitary channel current than observed in Na^+ saline. In six cells examined under this condition, four responded with a decrease in amplitude of the single channel current as shown in Fig. 3, while the others showed no change.

The concentration dependence of UTP in cells bathed in Na^+ saline was examined next. In the experiment of Fig. 4*A*, a stepwise increase in the concentration of UTP (10 to 100 nM) revealed a threshold level at approximately 30 nM. This threshold concentration varied considerably from cell to cell, and, in other experiments, an initial response was not detected until the concentration of nucleo-

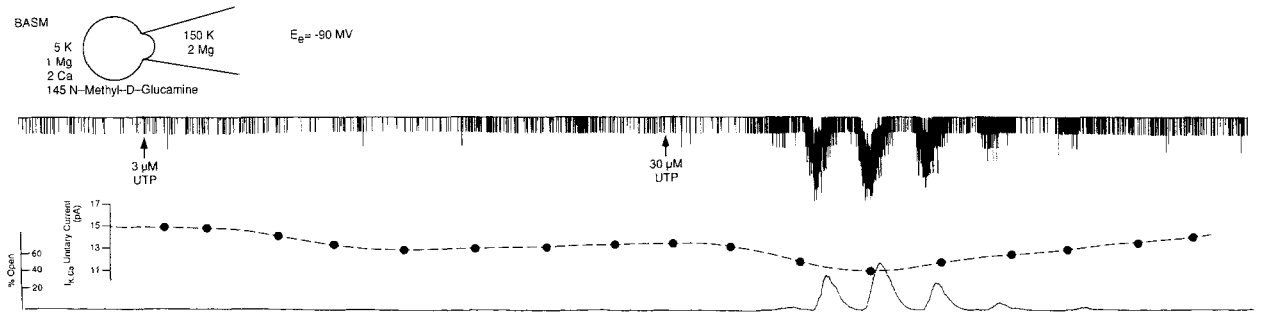

Fig. 3. Effect of UTP on aortic smooth muscle in Na^+-free saline. Substitution of N-methyl-D-glucamine for Na^+ prior to application of UTP blocked the large increase in Maxi-K channel activity typically observed for 3.0 μM UTP. However, a change in Maxi-K channel unitary current amplitude was detected (plot), but it showed a transient decrease, rather than the increase that always occurs in cells bathed in Na^+ saline. An oscillatory activation of the channels similar to that evoked in Na^+ saline by 3.0 μM UTP was noted when 30 μM UTP was applied. This effect was accompanied by a larger transient decrease in amplitude of the unitary channel current.

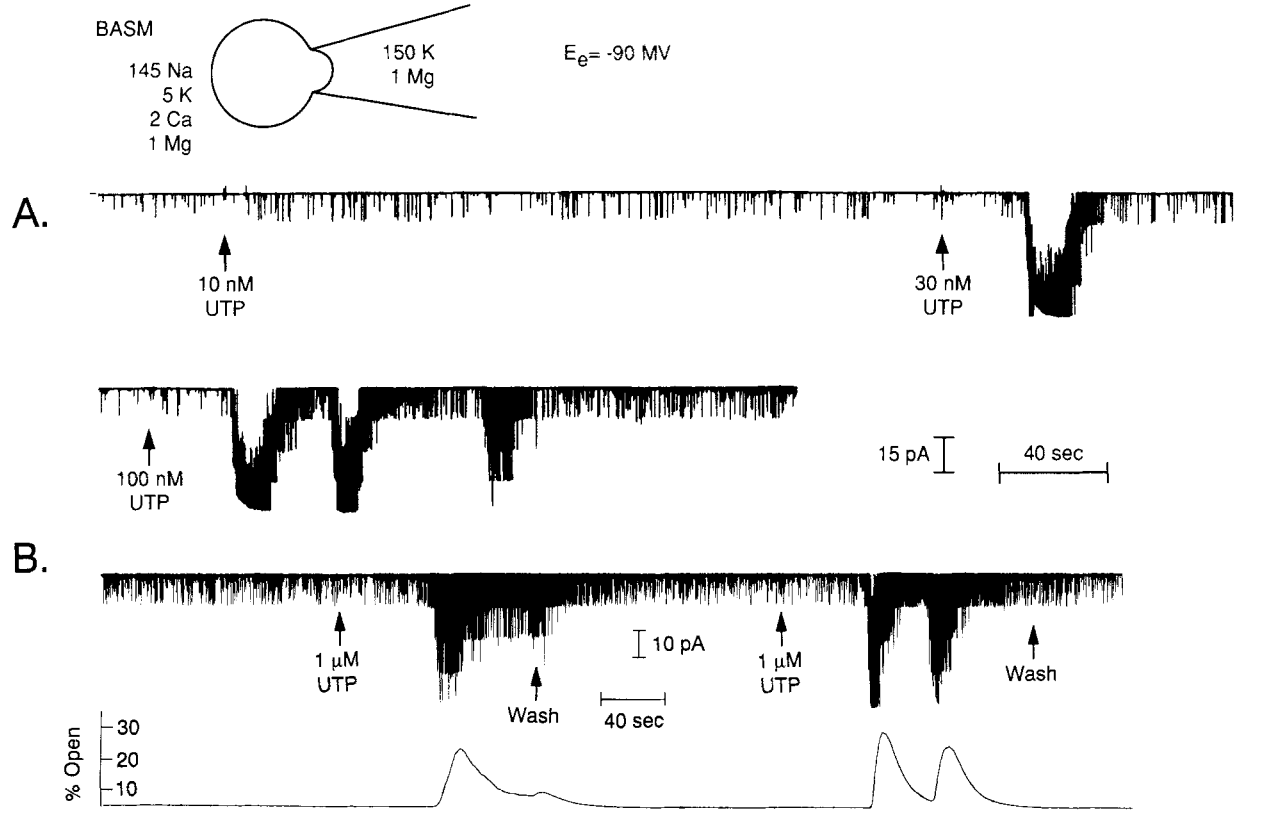

Fig. 4. Concentration dependence and time course of the UTP effect in aortic smooth muscle. Cells bathed in Na^+ saline may respond to UTP at concentrations as low as 30 nM, and the nucleotide-induced responses display refractoriness. A stepwise increase in UTP levels from 10 to 100 nM showed an initial response in stimulating Maxi-K channel activity at 30 nM, and a longer lasting oscillatory response at 100 nM (*A*). In another cell under the same conditions (*B*), it was possible to elicit UTP responses with similar percent channel open times with a 4 min interval between UTP applications. Percent channel open time decreases for the second response at intervals shorter than 4 min for both UTP and ATP, or for combinations of the nucleotides ("cross desensitization"; *see* Fig. 5).

tide approached 300 nM. In view of this variability in the sensitivity to UTP, μM levels of nucleotide were used in subsequent experiments. It was also essential for experimental protocol design to determine the length of time necessary between UTP exposures in order to obtain a comparable increase in channel activity. For example, in Fig. 4*B*, the percent open time during the first and second exposures to 1.0 μM UTP was nearly equal with a 4 min intervening period. In cells bathed in Na^+ saline, a

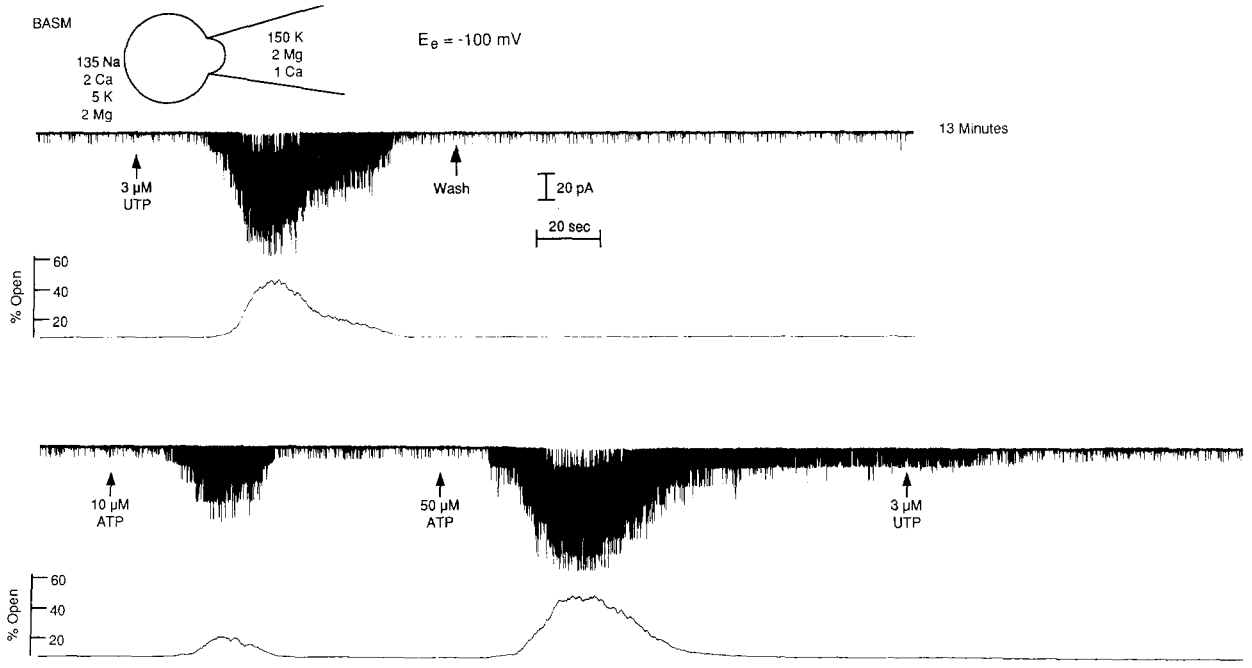

Fig. 5. Effect of ATP on intracellular Ca^{2+} concentrations in bovine aortic smooth muscle. ATP is less effective than UTP in causing an increase in Maxi-K channel activity in bovine aorta cells. ATP at 10 μM elicited a smaller response than that recorded in 3.0 μM UTP. It was necessary to increase the ATP concentration to 50 μM in order to obtain a similar increase in percent channel open time as that recorded in 3.0 μM UTP. Note that the final exposure to 3.0 μM UTP did not enhance Maxi-K channel activity, indicating "cross-desensitization" for these agonists.

5 min period was sufficient for full recovery of the response, but for cells in 150 mM K^+, times that approached 10 min were required to achieve this effect.

Effectiveness of Other Nucleotides on the Maxi-K Channel Response

In addition to UTP, several other nucleotides were examined for their effects on Maxi-K channel activity in cell-attached patches. The data of Fig. 5 indicate that the concentration of ATP required to elicit a similar increase in percent open time to that obtained with 3.0 μM UTP is above 10 μM, and close to 50 μM. An additional exposure of the cell to 3.0 μM UTP, approximately 2 min following 50 μM ATP, did not cause enhancement of channel activity. Figure 6 is a composite of recordings from two additional cells in which guanosine triphosphate (GTP) and cytosine triphosphate (CTP) are shown to be ineffective nucleotides in eliciting a Maxi-K channel response. In one cell (Fig. 6*A*), 150 μM GTP failed to induce a change in channel activity during a prolonged exposure (~5 min). Only after superfusing the cell with a saline containing 150 μM GTP plus 1.0 μM UTP, was a transient increase in channel activity noted. In a second cell (Fig. 6*B*), 500 μM CTP was without effect, while 50 μM ATP elicited a typical response noted for cells bathed in 150 mM K^+ saline. In this depolarized cell, the on-cell patch contained only two Maxi-K channels and the lack of change in amplitude of the unitary current is apparent. We have previously published data (Williams et al., 1988) which showed that adenine nucleotides (up to 1.0 mM) were without effect on Maxi-K channels in excised patches from these same cells when tested in either inside-out or outside-out membrane configurations. Guanine nucleotides were active, but only when applied to inside-out patches, and GMP was the only nucleotide that modified channel activity in the low μM range. In view of the effects of UTP on Maxi-K channels within on-cell patches, nine outside-out patches were exposed to UTP at concentrations ranging from 50 μM to 1.0 mM, and in all these patches UTP did not affect channel activity (*not shown*).

Dependence of UTP-induced Maxi-K Channel Activation on Intra- vs. Extracellular Ca^{2+}

To determine the source of Ca^{2+} responsible for Maxi-K channel activation after exposure of smooth muscle cells to UTP, we initially used protocols in which extracellular Ca^{2+} was removed. In the exper-

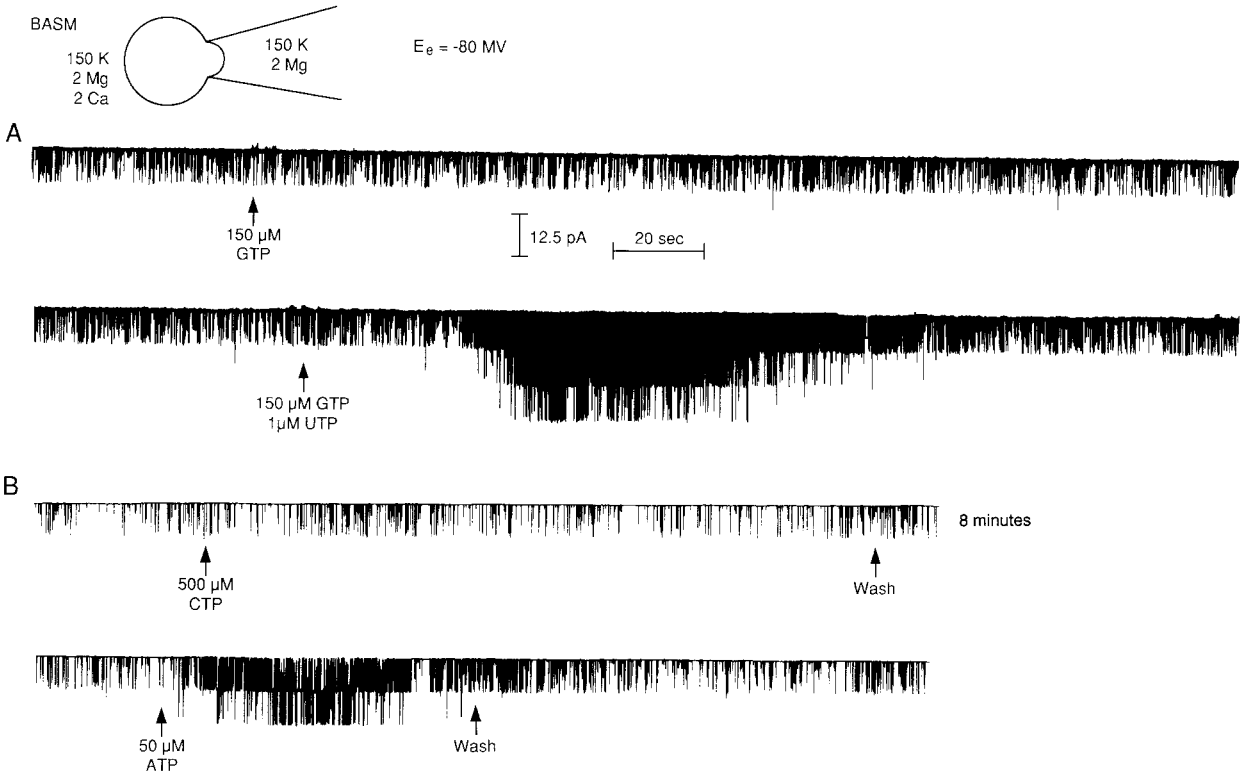

Fig. 6. Effect of GTP and CTP on intracellular Ca^{2+} concentrations in bovine aortic smooth muscle. GTP (*A*) and CTP (*B*) were two of the nucleotide triphosphates examined in bovine aorta cells exposed to depolarizing K^+ concentrations. Both agents were inactive in stimulating Maxi-K channel activity at concentrations ranging from 100 to 500 μM.

iment of Fig. 7, the cell was continuously washed with 150 mM K^+ saline containing 20 μM EGTA ("Ca^{2+}-free" conditions). Approximately 10 min of washing time was deleted following each line of recording. The first exposure to 10 μM UTP, after washing the cell for about 10 min with the "Ca^{2+}-free" saline, evoked a large increase in activity of the two channels present within the patch. The second exposure to 10 μM UTP caused a minimal increase in activity, and the final challenge with UTP did not elicit a response even though the concentration of nucleotide was increased to 30 μM. In other experiments with cells bathed in standard Na^+ saline (2.0 mM Ca^{2+}), Ca^{2+} entry blockers were used to evaluate whether the initial response to UTP was dependent upon Ca^{2+} influx. The agents tested were felodipine at 1.0 μM (n = 2) and La^{3+} at 50 and 100 μM (n = 2). Neither of these agents affected the initial UTP response. Next, the interaction between Ca^{2+} ionophore- and UTP-induced responses was investigated. The data of Fig. 8 were gathered from three cells, each one exposed first to UTP followed by treatment with either 2 or 3 μM A23187 after a variable interval of time. In Fig. 8*A*, there was a 12 min interval between application of 3.0 μM UTP and 2.0 μM A23187. The peak increase in percent open time evoked by each of these agents was comparable, although the time courses of their effects were different. The second cell (Fig. 8*B*) was also treated with 3.0 μM UTP before it was exposed to 2.0 μM A23187 after a 3 min interval, and here the peak response to the ionophore was about 20% of that elicited by UTP. In Fig. 8*C*, long-lasting oscillations in channel activity were evoked by a maximal UTP concentration (30 μM), but when this cell was challenged with 3.0 μM A23187 after a 4 min interval, there was no further enhancement in channel activity.

Evidence for G-Protein Mediation of Nucleotide-Induced Responses

In view of reports indicating that in some cell types the action of ATP and UTP to release intracellular-stored Ca^{2+} is mediated by a regulatory G-protein pathway (*see* Discussion), we performed a number of experiments following treatment of smooth muscle cells with pertussis toxin. In these experiments, A23187, whose action to elevate cytosolic Ca^{2+} is clearly not mediated by a G-protein mechanism, served as the control. Figures 9 and 10 are composed

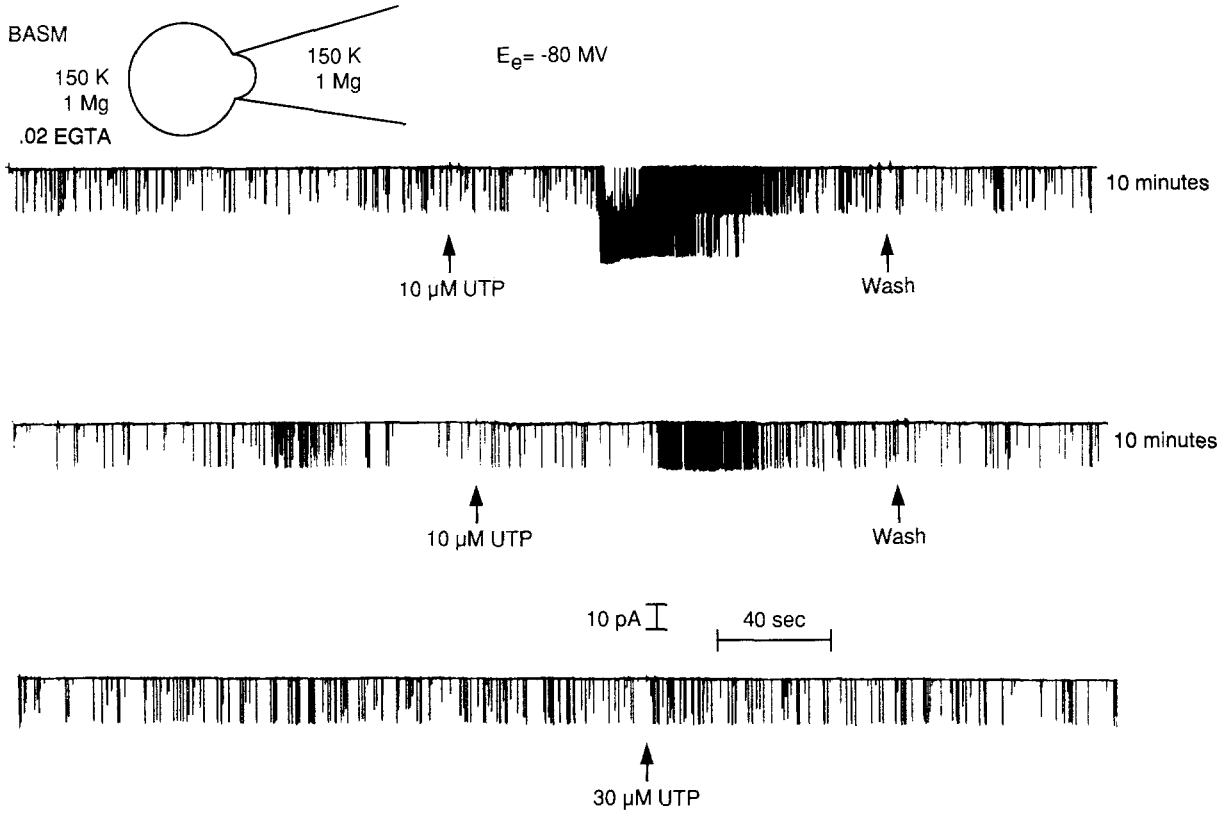

Fig. 7. Effect of removing extracellular Ca^{2+} on the UTP response in bovine aortic smooth muscle. UTP cannot elicit multiple responses in terms of activating Maxi-K channel activity in the absence of extracellular Ca^{2+}.

of data from cells that were treated with 20 ng/ml of pertussis toxin for *ca.* 20 hr before subjecting them to experimental conditions in which either nucleotides or A23187 were present. In Figure 9, an on-cell patch with one Maxi-K channel showed a minimal increase in percent open time (~10%) and no detectable change in unitary current amplitude upon perfusion with saline containing 50 μM ATP. After 9 min of washing, a challenge with 2 μM A23187 increased the open time of the Maxi-K channel to 65%. A similar experiment with UTP is shown in Fig. 10; 2 μM UTP was ineffective in activating Maxi-K channel activity, while 2 μM A23187 evoked an increase of 40% in the open time of the channel. The data obtained from 10 experiments using this protocol, but with different concentrations of nucleotides, are listed in the Table.

Effect of Modulators of Protein Kinase C

The data presented above on nucleotide-induced release of intracellular Ca^{2+} and the dependence on G-protein interactions suggest that UTP induces the formation of IP_3 which then serves as the messenger for intracellular Ca^{2+} release. Therefore, we examined the possibility that the time course of the nucleotide-induced response and/or that the recovery period before another nucleotide response can be elicited are modulated by protein kinase C (PKC) activity. The data of Fig. 11 demonstrate that phorbol myristate acetate (PMA), a PKC agonist, is a potent inhibitor of the UTP-induced increase in Maxi-K channel activity. A 3 min exposure of the cell to 10 nM PMA considerably reduced the response elicited by 3.0 μM UTP, and, after 10 min in 10 nM PMA, the response was totally blocked. A brief increase in channel activity was evoked, however, upon elevating UTP to 50 μM. A23187 (2 μM) enhanced channel activity after 15 min in 10 nM PMA, although to an apparently lesser extent than the initial response to 3.0 μM UTP. At 250 nM PMA (Fig. 12), total block of a 3.0 μM UTP response occurred after 5 min exposure to this agent, and 5.0 μM A23187 caused only a small percent increase in channel open time. In the same experiment, two control responses gave peak open times near 75%. In contrast to these results, a phorbol ester which is

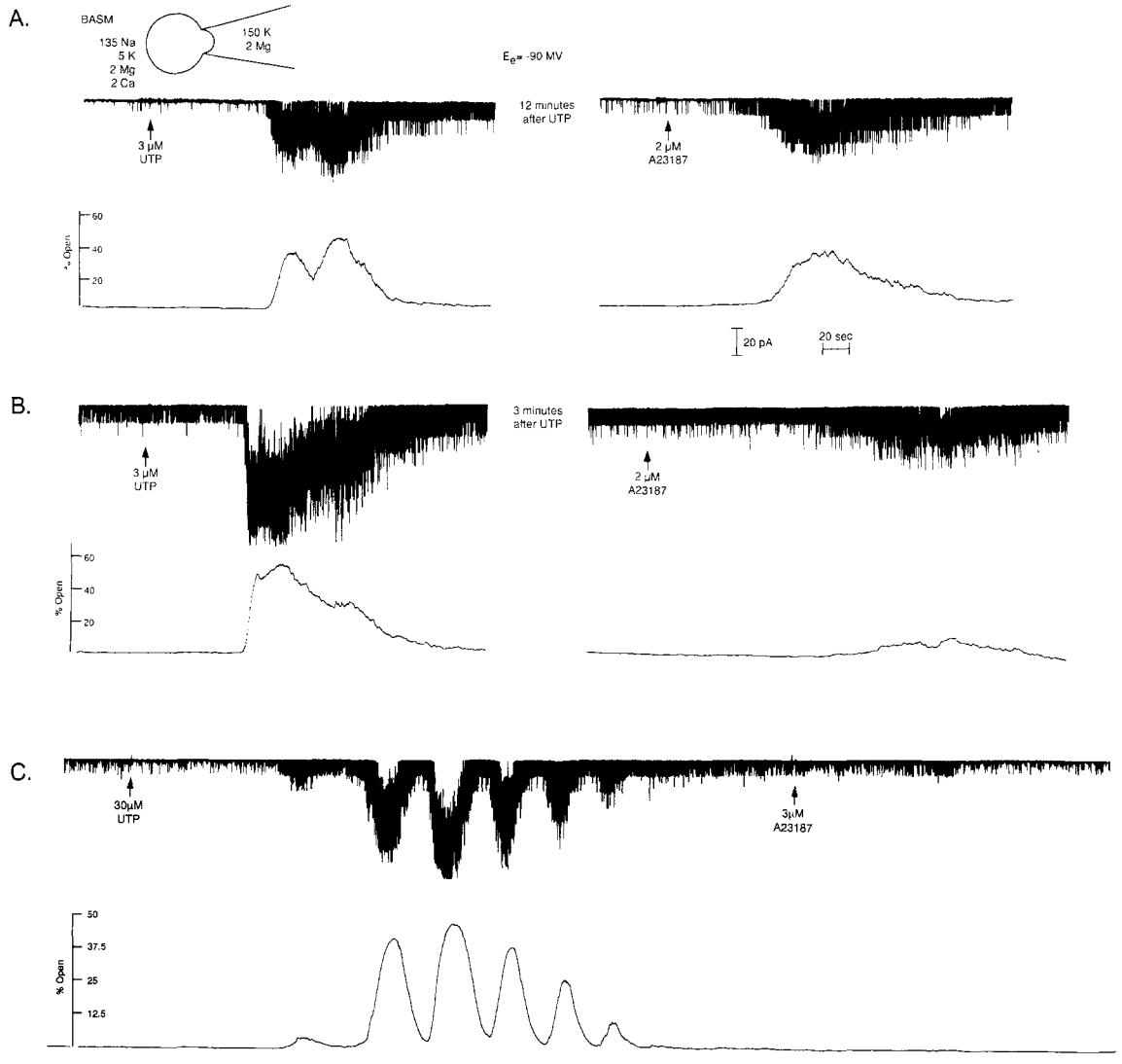

Fig. 8. UTP and A23187 demonstrate "cross-desensitization" in their ability to stimulate Maxi-K channel activity in bovine aortic smooth muscle. In *A* and *B*, the time interval between exposure of a bovine aorta cell to UTP and A23187 was progressively decreased and Maxi-K channel activity was monitored. In *C*, the concentration of UTP was elevated before subsequent application of ionophore.

inactive as an activator of PKC, 4 α-phorbol, 12,13-didecanoate, had no effect on either UTP- or A23187-induced responses, even at concentrations as high as 1.0 μM (*not shown*). Interestingly, PMA at a high concentration (500 nM) had no effect on the activity of Maxi-K channels within excised inside-out patches (n = 3).

A number of antagonists of PKC were also examined, and the results obtained with one of these (staurosporine) are shown in Fig. 13. In Fig. 13*A*, a large oscillatory burst of channel activity was initiated following superfusion of the cell with saline containing 3.0 μM UTP. In Fig. 13*B*, the cell was re-exposed to 3.0 μM UTP after pretreatment with 0.5 μM staurosporine for 8 min. During the pretreatment period the basal channel activity increased, but this was not observed in other experiments. A consistent finding in all experiments with staurosporine (n = 19: 0.1 to 200 μM concentration range), as well as with D-sphingosine (n = 3: 10 to 20 μM concentration range), H-7 (n = 13: 0.1 to 200 μM concentration range), and cremophor (n = 3: 10 μM) was a lack of effect of these agents on the UTP-induced Maxi-K channel responses. Furthermore, these antagonists of PKC did not prevent PMA from inhibiting the nucleotide-elicited responses. In Fig. 13*C*, 100 nM PMA was added to the superfusion medium that contained 0.5 μM staurosporine 10 min prior to the third application of 3.0 μM UTP, which evoked a minimal increase in channel activity as compared to that noted in Fig. 13*A* and *B*. In four experiments, UTP-induced responses were re-

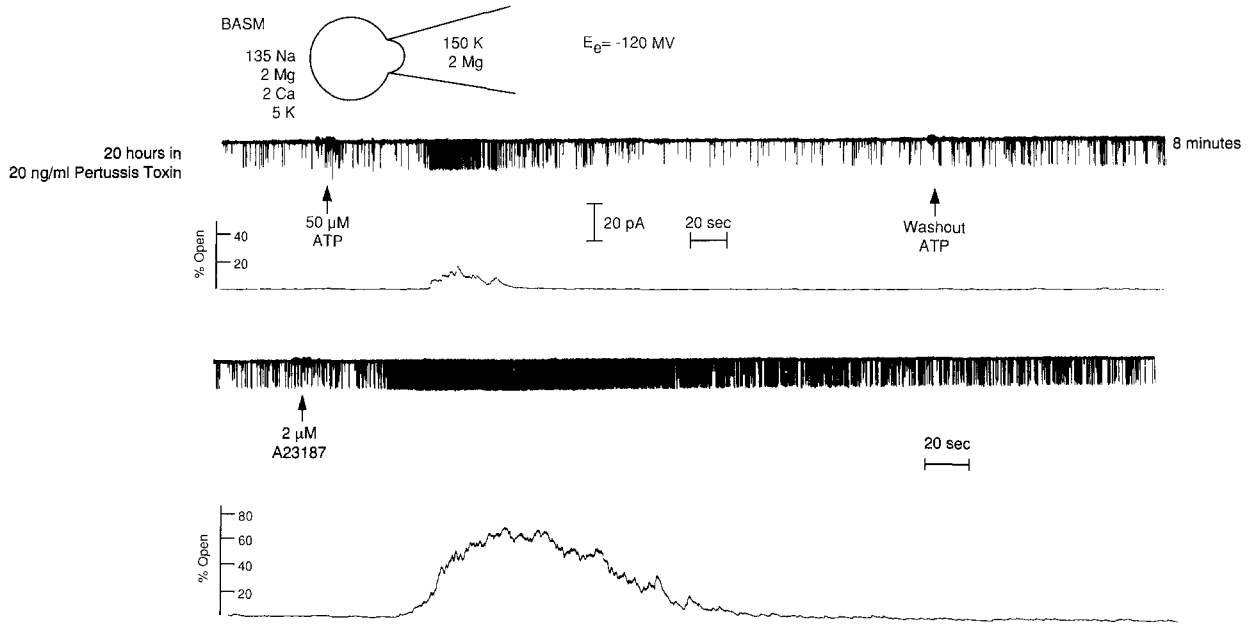

Fig. 9. Effect of pertussis toxin treatment on the ability of ATP to activate Maxi-K channels in bovine aortic smooth muscle. Bovine aorta cells treated with pertussis toxin show a reduced sensitivity to ATP for Maxi-K channel activation, but maintain their responsiveness to A23187.

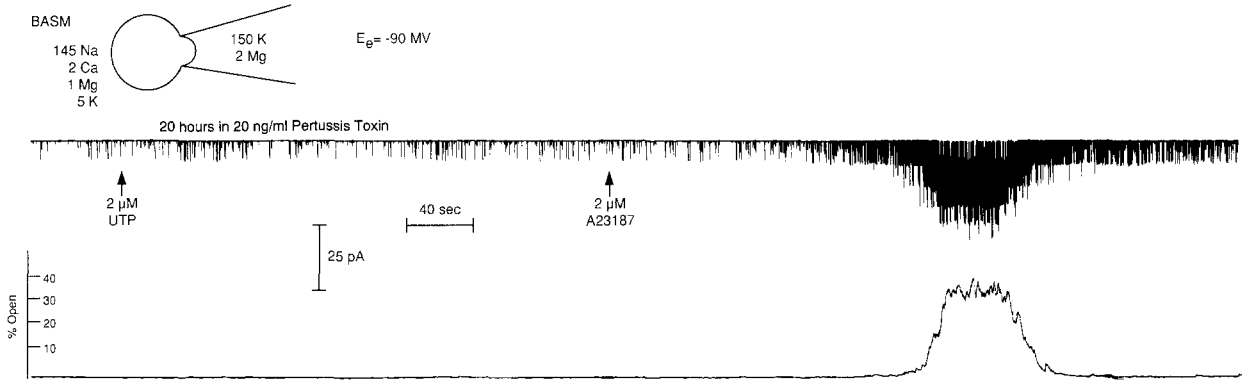

Fig. 10. Effect of pertussis toxin treatment on the ability of UTP to activate Maxi-K channels in bovine aortic smooth muscle. Pretreatment of bovine aorta cells with pertussis toxin decreases the sensitivity of Maxi-K channel activation to UTP. However, the effect of A23187 is not changed.

corded in cells pretreated with okadaic acid (100 to 300 nM). The use of this phosphatase inhibitor served as an additional test of the possible involvement of PKC and protein phosphorylation in determining the properties of the nucleotide-elicited responses. Okadaic acid, even after prolonged exposure periods (up to 30 min), did not modify either basal channel activity or the enhancement of activity caused by UTP treatment (*data not shown*).

Discussion

The results described in this study provide a mechanistic basis for the ability of UTP to cause contraction of bovine aortic smooth muscle strips. Namely, UTP binds to a putative "pyrimidinergic receptor" at the extracellular surface of the smooth muscle sarcolemma. Binding of ligand to this receptor elicits two effects: release of Ca^{2+} from intracellular stores and depolarization of the sarcolemmal membrane

Table. Effect of pertussis toxin treatment on nucleotide responses in bovine aortic smooth muscle cells

Nucleotide (μM)	Extracellular K^+ (mM)	Nucleotide/A23187 Response
2 UTP	5	0
5 UTP	150	0
10 UTP	150	0.09
10 UTP	150	0
10 UTP	5	0.25
10 UTP	150	0
10 UTP	150	0.20
10 UTP	5	0
50 ATP	5	0.15
100 ATP	150	0.065

Bovine aortic smooth muscle cells were treated overnight with pertussis toxin (20 ng/ml). They were then exposed to various nucleotides at different concentrations and the Maxi-K channel activity was recorded in cell-attached patches. These experiments were performed with either polarized or depolarized cells. After washing out the nucleotide, 2 μM A23187 was added and the percent open time response in nucleotide *vs.* ionophore treatment was compared.

due to activation of an inward current. Both processes modify the activity of Maxi-K channels within cell attached patches which can be used as a reporter to detect such changes under these conditions. Clearly UTP elevates cytosolic Ca^{2+} by releasing this ion from intracellular stores because the average percent open time of the Maxi-K channel transiently increases after exposure to nucleotide, and the effect is not blocked by removing extracellular Ca^{2+}. Furthermore, low concentrations of Ca^{2+} ionophores produce a similar effect. However, a major difference between the action of UTP and Ca^{2+} ionophores is that the former evokes an increase in the amplitude of unitary Maxi-K channel currents when measurements are made using cell-attached membrane patches. This change in amplitude results from the ability of UTP to depolarize the cell membrane by eliciting a transient macroscopic inward Na^+ current. The UTP-evoked current can be recorded using the perforated-patch configuration of whole-cell voltage clamp after exposure of cells to the nucleotide. UTP binding to its receptor primarily increases cell Ca^{2+} by emptying intracellular Ca^{2+} stores, but a minor contribution of influx through

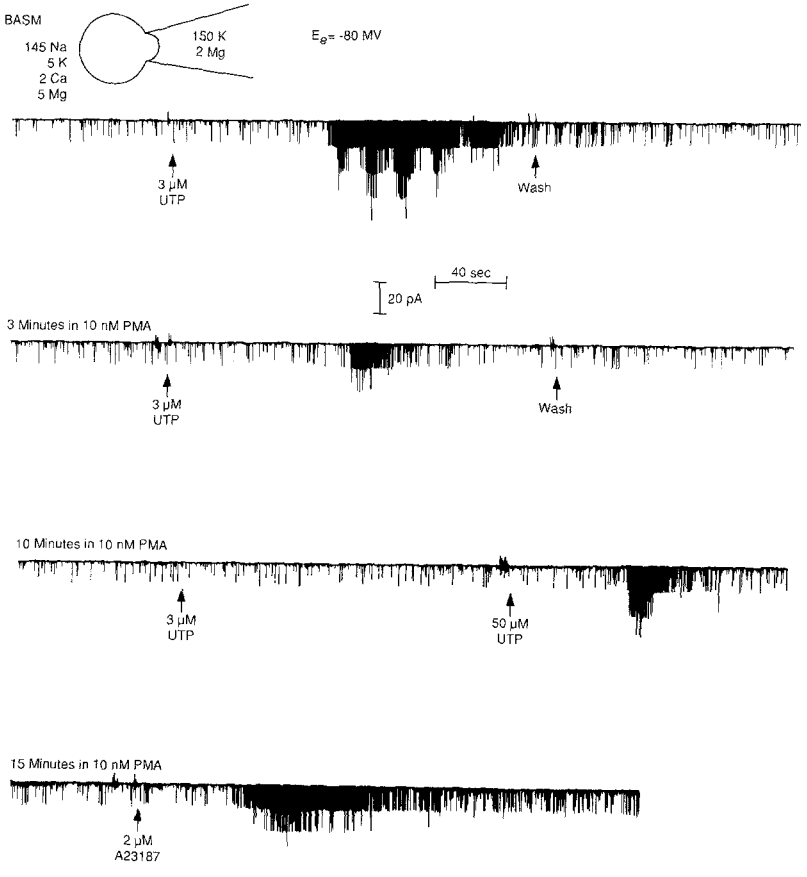

Fig. 11. Effect of phorbol myristate acetate on the ability of UTP and ionophore to activate Maxi-K channel activity in bovine aortic smooth muscle. The phorbol ester, PMA, at 10 nM blocks the increase in Maxi-K channel activity elicited by 3.0 μM UTP in bovine aorta cells. This agent also reduces the effects evoked by 2.0 μM A23187.

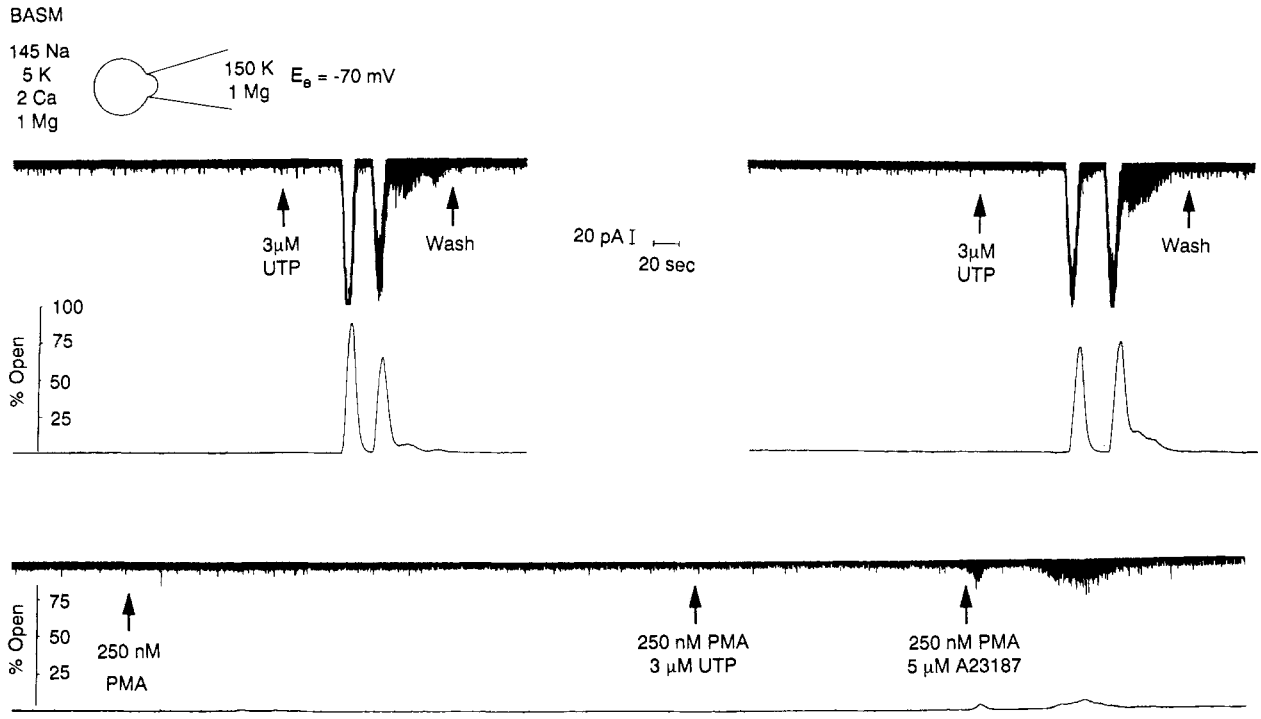

Fig. 12. Effect of phorbol myristate acetate on the ability of UTP and ionophore to activate Maxi-K channel activity in bovine aortic smooth muscle. High concentrations of PMA (250 nM) block both UTP- and A23187-induced Maxi-K channel activation within minutes (~5 min) in bovine aorta cells.

voltage-gated Ca^{2+} channels following cell depolarization cannot be dismissed. Both processes will promote contraction of aortic smooth muscle.

All attempts to identify the channel that conveys the UTP-mediated inward current, as well as the pathway which regulates the activity of this channel, were unsuccessful. However, channels which are Ca^{2+}-activated can be ruled out based on the following observations: (i) no inward current was recorded in the presence of a Ca^{2+} ionophore; (ii) the time course of the inward current differs from that of variations in intracellular Ca^{2+} concentrations; (iii) prolonged exposure of cells to the Ca^{2+} chelator, BAPTA/AM, did not prevent the increase in the inward current that is measured (*not shown*). Several experiments with excised membrane patches were performed to gain insight into the mechanism by which UTP activates these cation-selective channels. In experiments with outside-out membrane patches, UTP did not elicit additional channel activity. Furthermore, with inside-out membrane patches ($n = 3$), exposure of the inner membrane surface to IP_3 (0.5 to 5 μM) did not result in the appearance of channels activated by this second messenger. Some information was also gathered concerning the selectivity of this conductance pathway. A major component of the current is conveyed by Na^+, since following substitution of Na^+ by N-methyl-D-glucamine in the bathing medium, the cell membrane responded to UTP exposure with a transient hyperpolarization. In addition, unlike the inward currents recorded in rabbit ear artery smooth muscle following exposure to ATP (Benham, 1989), the percentage of the UTP-induced inward current conveyed by Ca^{2+} must be small. This conclusion is based on the finding that the increase in intracellular Ca^{2+} concentration is similar in the absence or presence of extracellular Ca^{2+}, following treatment of cells with UTP. Measurements of the reversal potential of the inward current for cells bathed in Na^+ saline gave values close to -10 mV. This finding, along with the lack of change in the Maxi-K channel unitary current amplitude noted in cells bathed in 150 mM KCl, indicate that this pathway must be monovalent cation selective. While the channels responsible for the cation-selective inward current and their mode of activation remain to be clarified, it is clear that the basis for the UTP-mediated enhancement of Maxi-K channel activity within on-cell patches is the result of two processes: (i) a rise in intracellular Ca^{2+} concentration and (ii) depolarization of the cell membrane. That the former is the major contributor to the increase in Maxi-K channel activity is demonstrated by the persistence and magnitude of the increases observed in cells that are bathed in 150 mM K^+ saline.

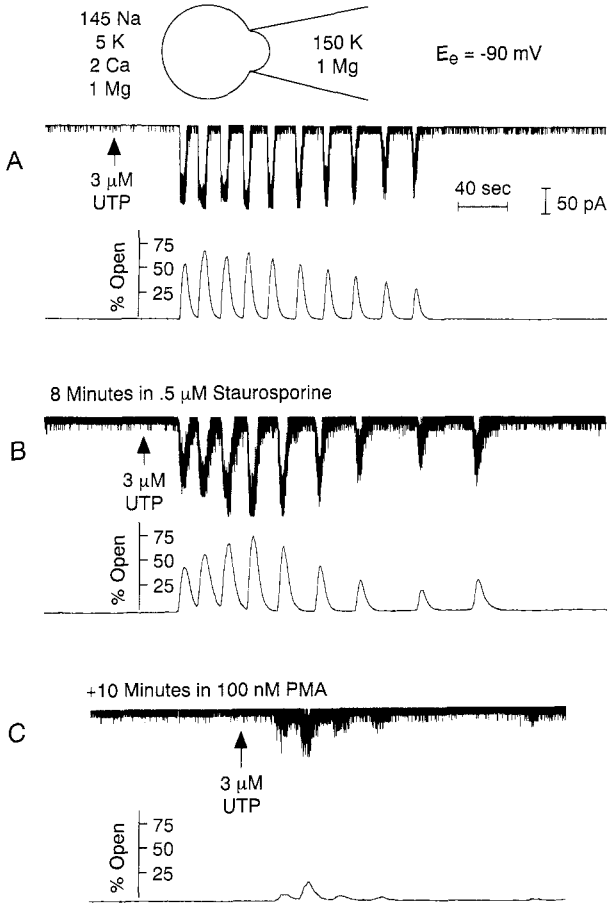

Fig. 13. Effect of a protein kinase C inhibitor on the ability of UTP and phorbol myristate acetate to modify Maxi-K channel activity in bovine aortic smooth muscle. Staurosporine (*B*), a blocker of PKC, did not reduce the oscillatory increase in channel activity elicited by 3.0 μM UTP (*A*), nor did it antagonize the action of PMA to block the responses to UTP (*C*).

The time course of the change in intracellular Ca^{2+} concentration evoked by UTP and the refractoriness of this response to subsequent challenges with nucleotide appear to be determined primarily by the availability of stored Ca^{2+}, and not by a desensitization of the pyrimidinergic receptor system. This conclusion is based upon the observation of "cross desensitization" which is noted to occur between UTP and A23187. That is, exposure of smooth muscle cells first to UTP can blunt the A23187 response, unless sufficient time is provided for the cells to recover. Since A23187 bypasses the nucleotide receptor system, the common factor must be release of Ca^{2+} from intracellular pools. In these experiments, ionophore must be applied after UTP, since cells only respond once to challenge with ionophore. This is apparently the result of the slow washout of ionophore following incorporation of this agent into cellular membranes. Taken together, these data support a scheme in which UTP mediates release of Ca^{2+} from intracellular stores, followed by the refilling of these stores before a second challenge with nucleotide is able to evoke another effect.

Despite the rise in intracellular Ca^{2+} concentration elicited by both agents, a characteristic of the UTP response which is clearly different from that of Ca^{2+} ionophores is the oscillations in Maxi-K channel activity induced by the nucleotide. The lack of oscillations in cells exposed to A23187 was surprising, since both ionophore and UTP release Ca^{2+} from intracellular pools. The evidence that ionophores release Ca^{2+} from intracellular compartments rather than promote the influx of extracellular Ca^{2+} is based on three findings: (i) the rise in intracellular Ca^{2+} concentration is transient; (ii) activation of Maxi-K channel activity occurs in the absence of external Ca^{2+}; (iii) data from "cross desensitization" experiments suggest that UTP and A23187 release Ca^{2+} from the same intracellular pool(s). While the mechanism for inducing the oscillatory behavior is unclear, the present data would seem to indicate that oscillations are not initiated by a rise in intracellular Ca^{2+} concentration since they only occurred with UTP (IP_3 signal), but not with A23187. However, this may be a hasty conclusion for several reasons. A23187 is not readily reversible, apparently maintaining the membranes responsible for sequestering intracellular Ca^{2+} pools in a state of high Ca^{2+} permeability, which would prevent Ca^{2+} cycling and the appearance of oscillations. Furthermore, in other types of cells, Ca^{2+} ionophores appear to preferentially modify the permeability of the surface membrane, thereby enhancing Ca^{2+} influx. Under this condition, Ca^{2+} ionophores have been noted to cause an oscillatory behavior (Wakui, Osipchuk & Petersen, 1990). In addition, skinned muscle fibers with intact sarcoplasmic reticulum respond to added Ca^{2+} with oscillations in tension under weak Ca^{2+} buffering, and in the complete absence of IP_3. Therefore, in view of the above observations, a rise in intracellular Ca^{2+} concentration may still be the common denominator (Petersen & Wakui, 1990) for initiating the oscillations in Maxi-K channel activity noted in this study.

The evidence that the UTP-elicited response involves a second messenger system which is responsible for signaling Ca^{2+} release is summarized by the data in Figs. 8 and 9 as well as in the Table. These results indicate that a pertussis toxin-sensitive G protein mediates the signal between the UTP receptor and a rise in intracellular Ca^{2+} concentration. This communication probably involves the phosphatidyl inositol pathway, since UTP has been reported to increase IP_3 levels in other cell types (Demolle, Lagneau & Boeynaems, 1988; Davidson et al., 1990;

Murrin & Boarder, 1992). While an in-depth characterization of the nucleotide binding site(s) must await the discovery of high affinity probes for this moiety, these data have led us to use the term "pyrimidinergic receptor" as the site of action of UTP. As expected, the effect of Ca^{2+} ionophores, which act directly on intracellular Ca^{2+} storage compartments, are not modified by toxin treatment. For this reason, the ionophore-induced responses were used as controls in the experiment with pertussis toxin to evaluate the availability of stored Ca^{2+} following overnight exposure of cells to this agent.

Since the UTP binding site appears to affect cellular activities, at least in part, via its G-protein coupling to the phosphatidyl inositol system, the possibility was raised that PKC modulates the IP_3-induced changes in intracellular Ca^{2+} concentration (*see* Llano & Marty, 1987). Initial findings with 10 nM PMA indicated that stimulation of PKC caused inhibition of the UTP-induced increase in intracellular Ca^{2+}, and the increase in amplitude of the Maxi-K unitary channel current. The inactive phorbol ester (4α-phorbol 12, 13-didecanoate) at concentrations up to 500 nM did not modify the UTP response. Also at 10 nM PMA, there was only a minimal effect on Maxi-K channel activity elicited by application of 2–3 μM A23187. However, high concentrations of PMA (100 to 250 nM) greatly reduced the increase in intracellular Ca^{2+} concentration evoked by A23187 as previously noted in endothelial cells by Flavahan, Shimokawa and Vanhoutte (1991). If PMA is a selective PKC agonist, then the above data suggest that PKC affects the Ca^{2+} available for release from intracellular pools. Yet experiments with inhibitors of PKC do not support this conclusion. Staurosporine (at concentrations up to 0.5 μM), as well as other inhibitors of PKC, did not modify the variations in intracellular Ca^{2+} concentrations detected in the absence of PMA, nor did they antagonize the ability of PMA to inhibit the UTP-induced elevation of Ca^{2+}. While PMA does not appear to be acting via PKC, its net effect in bovine aortic smooth muscle cells is to inhibit the release of stored Ca^{2+}. This conclusion could be questioned if PMA were to directly modify Maxi-K channels. However, this does not appear to be the case. PMA did not effect basal Maxi-K channel activity in either on-cell or excised membrane patches. Moreover, Maxi-K channel activity within inside-out patches excised from cells treated with PMA, and which no longer display a UTP response, maintained their normal sensitivity towards Ca^{2+}.

In view of the above discussion, a mechanistic scheme describing the coupling of the putative "pyrimidinergic receptor" to second messenger systems must be limited. With phosphatidyl inositol providing messengers, only the branch generating IP_3 appears to play a role in determining the time course and magnitude of intracellular Ca^{2+} concentration variations. In addition, the "pyrimidinergic receptor" appears to be coupled to a cation-selective channel, either directly or via a pertussis toxin-sensitive G protein, thus accounting for the UTP-induced inward current and membrane depolarization. Together, these pathways converge to elicit smooth muscle contraction.

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