Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: modulation by BK channels

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The urinary bladder has two principal functions in humans and animals. The first is the appropriate storage of the produced urine, and the second is the effective voiding of it. The latter requires coordinated contraction of the urinary bladder detrusor smooth muscle and relaxation of the muscles located in the internal and external urinary bladder sphincters. In adults, this process is under voluntary control and includes the interaction of neuronal and smooth muscle-specific mechanisms. The contractile state of smooth muscle involves the functional interplay of multiple processes, including neurotransmitter release, intracellular Ca2+ signaling, and ion channel regulation of membrane excitability. Malfunction at any one of these levels can lead to disturbed voiding symptoms like overactive bladder and urinary incontinence.

The urinary bladder smooth muscle (UBSM) is regulated by the parasympathetic nervous system, and this is the primary event that triggers voiding of the stored urine. Upon activation, parasympathetic nerve varicosities release the two neurotransmitters acetylcholine (ACh) and adenosine triphosphate (ATP), which bind to muscarinic (M3) and purinergic (P2X1) receptors, respectively, to cause UBSM contraction (25, 39). According to our current knowledge, P2X1Rs play a significant role in nerve-induced UBSM contraction in rodents (5), but M3 ACh receptors (AChR) play an important role in mice as well (8, 25). In humans, the AChR may be the more prominent receptors involved (4, 34), although an increased P2X1R-dependent signaling may contribute significantly, depending on the pathophysiological state of the tissue or experimental conditions (21, 28, 29, 35).

The large-conductance voltage- and Ca2+-sensitive potassium (BK) channel plays a major role in controlling UBSM contractility (16, 27, 38). BK channels are activated by membrane potential depolarization, as well as by increases in intracellular Ca2+ concentration ([Ca2+]i) (3, 9). Opening of BK channels is stimulated by local Ca2+ release events throughryanodine receptors (“Ca2+ sparks”) and may also be regulated by Ca2+ entry through voltage-dependent Ca2+ channels (VDCCs) or P2X1Rs (18, 19). Activation of the BK channel is involved in the repolarization of the action potential and the regulation of the resting membrane potential of UBSM (14). In a recent study, our laboratory demonstrated that UBSM from mice lacking the gene mSlo1 (Slo−/−) for the pore-forming α-subunit of the BK channel lack functional BK channels and exhibit increased phasic and nerve-evoked contractions, along with a change in the urination pattern (27). Our laboratory further showed that these mice exhibit increased bladder pressures and urine leakage (38). By using transmural electrical field stimulation (EFS), we found that UBSM strips from Slo−/− mice exhibit a left shift in frequency dependence, leading to higher contractile forces at lower stimulation frequency (38).

The contribution of nerve stimulation evoked cholinergic (ACh) and purinergic (ATP) signals to UBSM contractility in mouse is not known. Purinergic signaling acts through increase in UBSM excitability through stimulation of P2X1-R cation channels. Cholinergic signaling, via M3 receptors and Gq, increases inositol 1,4,5-trisphosphate and PKC activity, which...
has multiple effects, including increasing excitability (1). Thus, since purinergic signaling acts through an electrical mechanism, one might predict that it would be engaged at lower stimulation frequencies. Indeed, we found in mouse UBSM that purinergic-mediated force generation occurs at lower stimulation frequencies than cholinergic stimulation. Furthermore, we examined the origin of the left shift toward lower stimulation frequencies in Slo−/− mice by desensitizing and blocking the purinergic receptors with α,β-methylene ATP and suramin and by blocking the muscarinic AChRs with atropine. We found that both signaling pathways are enhanced at lower frequencies in the absence of BK channel activity. Interestingly, in addition to the left shift in Slo−/− UBSM frequency dependence, the purinergic transmission is reduced at higher frequencies, whereas the muscarinic transmission is increased. These results imply that both cholinergic- and purinergic-mediated force generation is modulated by UBSM excitability and that BK channel activity differentially modulates the outcomes of these pathways.

**METHODS**

**Tissue preparation.** The targeted disruption of the Slo−/− gene has been previously described (27). Male and female Slo+/+ and Slo−/− mice (12–18 wk old; ~25 g body weight) were euthanized with an overdose of an intraperitoneal injected pentobarbital (150 mg/kg body wt), according to the Institutional Animal Care and Use Committee of the University of Vermont. Bladders were removed and placed immediately in ice-cold dissection solution (in mM: 80 monosodium glutamate, 55 NaCl, 6 KCl, 10 glucose, 10 HEPES, 2 MgCl2, pH 7.3, adjusted with NaOH). No difference in bladder weight was observed (~20 mg; P > 0.05).

**Immunostaining.** Bladders were cleaned and dissected free of the urothelium and the suburothelium, and the UBSM layer was cut into 6–10 pieces. The pieces were incubated 5 min with the VDCC blocker nifedipine (1 μM; Sigma) to relax the UBSM and then stretched and fixed 45 min in PBS (Richard Allan Scientific) containing 4% formaldehyde (Polyscience). After washing, blocking, and permeabilization of the tissue in PBS with 4% normal goat serum (Jackson Immunoresearch) and 0.5% Triton X-100 (Sigma), it was incubated with the primary antibodies rabbit-anti-BK (1:1,000; Alomone Laboratories) and guinea pig-anti-protein gene product 9.5 (PGP9.5) (1:1,000; Chemicon) for 24 h at room temperature. After being washed three times with PBS/normal goat serum/Triton X, the UBSM pieces were incubated with the secondary antibodies anti-rabbit-Cy3 (1:400; Chemicon) and anti-guinea pig-FITC (1:50; Vector Laboratories) for 15 min at room temperature. The UBSM pieces were mounted with Cityfluor mounting media (Cityfluor), and images were acquired using a LSM 510 META Confocal Laser Scanning Imaging System from Zeiss.

**Contractility studies.** Force measurements were performed and analyzed as in prior publications (16, 17, 38). Briefly, the UBSM layer was dissected free of the urothelium and suburothelium, and the UBSM layer was cut into eight strips (2–3 mm wide and 5–7 mm long). One-half of the strips from three Slo−/− and three Slo+/+ mice were used for purinergic experiments, and one-half were used for cholinergic experiments. This provided 12 strips for each genotype and condition. Occasional overstretching of strips led to nonresponsiveness to EFS, which diminished n values to <12. Furthermore, iberiotoxin was applied to one-half of those 12 strips before the second stimulation, and one-half of them were untreated. This led to an average n value of 6 for each genotype and condition. Force production of strips was then measured using a MyoMed myograph (MED Associates, St. Albans, VT), and EFS parameters were as defined (16). Frequency-response curves were created by measuring the EFS-induced contraction amplitude at stimulus frequencies of 0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz. Pulse amplitude was 20 V, and polarity was reversed for alternating pulses. Pulse width was 0.2 ms, and stimulus duration was 2 s. Stimuli were applied every 3 min using a model PHM-152V stimulator (MED Associates). After the first frequency-response curve was generated, the UBSM strips were washed three times. Fifteen minutes after the final wash, various pharmacological compounds were added directly to the tissue bath. Strips were incubated in the presence of test compounds for 15 min, and then a second frequency-response curve was generated using the same EFS parameters as before. The used compounds were iberiotoxin (100 nM; Peptides International), atropine (1 μM), α,β-methylene ATP (10 μM), and suramin (10 μM; all from Sigma). Data were analyzed and presented using MyoMed (MED Associates), Mini-Analysis (Synaptosoft), Origin (OriginLab), Prism (GraphPad), and CorelDraw (Corel) software. Statistical comparisons were made using paired or unpaired t-tests, as applicable, and data are expressed with standard errors. A P < 0.05 was deemed significant.

**RESULTS**

**BK channel is expressed in UBSM but not in the innervating neurons.** It is well known that the BK channel is expressed in virtually all types of smooth muscle and in neurons of the central nervous system, and deletion of its gene leads to elevated blood pressure (32), urinary incontinence (27, 38), erectile dysfunction (41), and an ataxic phenotype (27, 33). However, it is not known whether this channel is also expressed in nerves that innervate the urinary bladder. In the absence of nerve stimulation, contractions to exogenous application of cholinergic and purinergic agonists were enhanced by blocking the BK channel with iberiotoxin or ablation of the Slo gene, suggesting a smooth muscle site of action (38). Nonetheless, a contribution of BK channels to parasympathetic nerve activity in the UBSM strips could not be excluded. To address this issue further, immunohistochemistry on whole mount urinary bladder detrusor was performed, using a BK channel-specific antibody, as well as an antibody for PGP9.5 as an axonal marker (10). Figure 1A shows the expression pattern of the BK channel in UBSM from wild-type (WT) mice (left) that was absent in Slo−/− mice [knockout (KO); right], supporting the specificity of the antibody. Nerve fibers in WT, such as those shown in Fig. 1B, detected by PGP9.5 antibodies, were not stained with the BK channel-specific antibody. Double-staining for PGP9.5 (green) and BK channel (red) showed no coexpression (Fig. 1C). The small number of yellow areas in nerve fibers results from the intensive red fluorescence bleeding through the green channel and not from coexpression. This result indicates that the BK channel is not expressed in efferent nerves in the UBSM strips and supports our previous findings that functional effects of BK channel block or deletion reside in the smooth muscle.

**Blocking of muscarinic AChRs with atropine reduced EFS-induced contractions in UBSM from Slo+/+ and Slo−/− mice.** UBSM contractions were characterized in response to increasing frequencies of transmural nerve stimulation, as shown previously in WT mice (16). In the present study, the regulation of EFS-induced contractions by BK channels was assessed by performing frequency-response curves in each UBSM strip from Slo+/+ (WT) and Slo−/− (KO) mice. By adding atropine to the bath before the second frequency-response curve, the muscarinic AChR and its downstream signaling pathway were blocked.
In WT UBSM strips, atropine had no significant effect at frequencies <7.5 Hz, but reduced the force by 40–60% at frequencies between 7.5 and 50 Hz (Fig. 2, A and B, left). This is consistent with other studies indicating that muscarinic receptors have a more prominent role at higher frequencies (5). In strips from KO mice, however, atropine had significant effects at a frequency of 2 Hz and reached 65–70% force inhibition at frequencies of ≥20 Hz (Fig. 2, A and B, right). The significant effects at lower frequency indicate a possible left shift in the contractile response to ACh and/or ATP release from nerve varicosities, which is demonstrated in Fig. 4 and related results. In the presence of atropine, blocking the BK channel in WT strips with the channel’s specific inhibitor iberiotoxin led to a dramatic increase in contractility over the whole frequency range (Fig. 2C, left), an effect that was absent in KO strips (Fig. 2C, right). In control experiments, where no iberiotoxin was added to the strips before the next stimulation, no change in the frequency-response curve was observed. Furthermore, blocking the BK channel in WT strips led to a characteristic frequency-response curve similar to the one from KO strips, with maximum force developed at stimulation frequencies <20 Hz (compare Fig. 2C, left, to Fig. 2B, right).

Desensitizing and blocking purinergic receptors with α,β-methylene ATP/suramin reduced EFS-induced contractions in UBSM from Slo+/+ and Slo−/− mice. To probe the role of purinergic-receptor signaling, α,β-methylene ATP and suramin were added to the bath before the second frequency-response curve. These drugs are generally used to activate and then desensitize and to block purinergic receptors and inhibit its downstream signaling pathway.

In UBSM strips from WT mice, the inhibition of purinergic receptors led to significant force reduction within the whole tested frequency range, except the highest 50 Hz (Fig. 3, A and B, left). This effect at low-stimulation frequencies is consistent
Fig. 2. Effects of atropine (Atr) on nerve-evoked urinary bladder smooth muscle (UBSM) contractions in Slo+/− and Slo−/− mice. A: representative recordings of nerve-mediated contractions from a WT (left) and a KO (right) mouse before and after treatment with Atr, with and without iberiotoxin (IbTX). Stimulations were delivered at frequencies of 0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz. B: frequency-response curves of UBSM strips from WT (left) and KO (right) mice in the absence and presence of Atr. C: electrical field stimulation (EFS)-induced contraction amplitude in the presence of Atr and IbTX expressed as a percentage of the corresponding control amplitude in the absence of Atr and IbTX. Ctrl, time control without adding IbTX. n = Number of strips from 3 WT and 3 KO mice. *P < 0.05.
Fig. 3. Effects of α,β-methylene ATP and suramin (Sur) on nerve-evoked UBSM contractions in Slo+/− and Slo−/− mice. A: representative recordings of nerve-mediated contractions from a WT (left) and a KO (right) mouse before and after treatment with α,β-methylene ATP and Sur, with and without IbTX. Stimulations were delivered at frequencies of 0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, and 50 Hz. B: frequency-response curves of UBSM strips from WT (left) and KO (right) mice in the absence and presence of α,β-methylene ATP and Sur. C: EFS-induced contraction amplitude in the presence of α,β-methylene ATP and Sur and IbTX expressed as a percentage of the corresponding control amplitude in the absence of drugs. n = Number of strips from 3 WT and 3 KO mice. *P < 0.05.
and B frequencies, indicating a very important role of ATP (Fig. 3, in KO UBSM strips also reduced the force significantly at low again with older studies (5). Inhibiting the purinergic receptors of nonpurinergic signaling at high stimulation frequencies in KO UBSM. In the presence of α,β-methylene ATP and suramin, blocking the BK channel in WT strips with iberiotoxin led to a dramatic increase in contractility at frequencies of ≥3.5 Hz (Fig. 3C), an effect that was absent in KO strips. In control experiments, no iberiotoxin was added to the strips before the next stimulation, and therefore no change in the frequency-response curve was observed.

**Dissection of cholinergic- and purinergic-mediated force in UBSM from Slo^{+/+} and Slo^{-/-} mice.** To separate the muscarinic receptor-mediated (atropine-sensitive) force from the purinergic receptor-mediated (α,β-methylene ATP/suramin-sensitive) force, the results from the first two sets of experiments were used (Figs. 1 and 2). To obtain muscarinic- and purinergic-mediated force, either the atropine-sensitive or the α,β-methylene ATP/suramin-sensitive frequency-response curves were subtracted from the corresponding control frequency-response curves. Figure 4A illustrates the force induced by the two neurotransmitters in strips from WT and KO mice. Cholinergic- and purinergic-mediated contractions are illustrated in the top and bottom panels of Fig. 4, respectively. In WT strips, the cholinergic-mediated force increased progressively over the stimulation frequency, with the steepest slope between 2.5 and 20 Hz. Above 20 Hz, the response curve flattened. In KO strips, the cholinergic force was greater at all frequencies >3.5 Hz, and maximum force at 50 Hz was 1.8-fold greater than the WT (Fig. 4A, top). In contrast, purinergic-mediated force increased steeper at lower frequencies than cholinergic-mediated force and was maximal at ~10–15 Hz. In KO strips, purinergic force was greater than in the WT at low frequencies (<7.5 Hz) and exhibited a sharper decline in force >10 Hz. Indeed, between 15 and 40 Hz, purinergic-mediated force was lower in KO than WT strips (Fig. 4A, bottom). In all cases, purinergic-mediated force was greater than cholinergic-mediated force <10 Hz and in WT even <20 Hz. Above 20 Hz, no difference between cholinergic and purinergic force was observed in WT strips. However, in KO strips, maximal force generation was significantly greater for cholinergic than purinergic pathways (20–50 Hz).

The blocker of BK channels, iberiotoxin, shifted the force-frequency relationship to the left in WT for both cholinergic (top) and purinergic-mediated (bottom) contractions (Fig. 4B). Iberiotoxin shifted the midpoints of cholinergic and purinergic force-frequency relationships from 19.09 ± 1.7 Hz to 12.1 ± 1.43 Hz (P < 0.05) and from 7.78 ± 0.47 Hz to 4.77 ± 0.42 Hz (P < 0.01), respectively (Table 1). Ablation of gene for the BK channel had an effect similar to iberiotoxin (Fig. 4C). In KO strips, the midpoints of cholinergic and purinergic force-frequency relationships were shifted from 19.09 ± 1.7 Hz to 9.71 ± 0.98 Hz (P < 0.01) and from 7.78 ± 0.47 Hz to 3.26 ± 0.31 Hz (P < 0.01), respectively (Fig. 4C, top and bottom and Table 1). In KO strips, iberiotoxin had no effect on cholinergic (P = 0.35) or purinergic (P = 0.15) force-frequency relationships at every frequency (Table 1).

**DISCUSSION**

Frequency encoding of cholinergic and purinergic signaling. It is known that both cholinergic (ACh) and purinergic (ATP) signaling pathways play an important role in UBSM contraction in rats (5, 22), rabbits (7), and guinea pigs (2, 5). We found that, in the WT mouse, purinergic signaling gener-
Fig. 5. Negative feedback regulation of nerve-evoked UBSM contractions by BK channels. Dashed lines illustrate negative feedback regulation of membrane potential (Em) mediated by BK channels to limit contractility. Lack of the channel in KO UBSM increases Em, depolarization and all downstream events (shown by bigger fonts and arrows). For simplicity, the different downstream signaling pathways of ACh and ATP that lead to depolarized Em, increased Ca\(^{2+}\) concentration, and contraction have not been shown. depol., depolarization; [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; VDCC, voltage-dependent Ca\(^{2+}\) channel.

Table 1. Effects of blocking cholinergic and purinergic pathways on contractile force and frequency-dependence in WT and KO urinary bladder smooth muscle strips

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + Atropin</th>
<th>WT + Atropin + IbTX</th>
<th>KO</th>
<th>KO + Atropin</th>
<th>KO + Atropin + IbTX</th>
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<tbody>
<tr>
<td>Maximum contractile force, mN</td>
<td>14.21 ± 1.78</td>
<td>7.05 ± 1.18*</td>
<td>11.46 ± 1.35†</td>
<td>15.27 ± 2.34</td>
<td>6.91 ± 0.85*</td>
<td>7.19 ± 1.09‡</td>
</tr>
<tr>
<td>Frequency of maximum contractile force, Hz</td>
<td>50</td>
<td>50</td>
<td>12.5</td>
<td>50</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>50% Contraction frequency, Hz</td>
<td>9.34 ± 0.34</td>
<td>7.78 ± 0.47*</td>
<td>4.77 ± 0.42†</td>
<td>5.01 ± 0.51‡</td>
<td>3.26 ± 0.31*</td>
<td>2.74 ± 0.33‡</td>
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<tr>
<th></th>
<th>WT</th>
<th>WT + α,β-Methylene ATP and Suramin</th>
<th>WT + α,β-Methylene ATP and Suramin + IbTX</th>
<th>KO</th>
<th>KO + α,β-Methylene ATP and Suramin</th>
<th>KO + α,β-Methylene ATP and Suramin + IbTX</th>
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<tr>
<td>Maximum contractile force, mN</td>
<td>15.11 ± 1.98</td>
<td>10.06 ± 1.78*</td>
<td>15.12 ± 3.64</td>
<td>17.97 ± 2.29</td>
<td>15.06 ± 2.36</td>
<td>11.67 ± 2.89</td>
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<tr>
<td>Frequency of maximum contractile force, Hz</td>
<td>50</td>
<td>50</td>
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<tr>
<td>50% Contraction frequency, Hz</td>
<td>10.25 ± 0.62</td>
<td>19.09 ± 1.7*</td>
<td>12.1 ± 1.43†</td>
<td>4.8 ± 0.61‡</td>
<td>9.71 ± 0.98*</td>
<td>12.04 ± 2.41*</td>
</tr>
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Values are means ± SE, except for the frequency of maximum contractile force. WT, wild type; KO, knockout; IbTX, iberiotoxin. *P < 0.05 vs. untreated. †P < 0.05 vs. absence of IbTX. ‡P < 0.05 vs. WT.
As well as an increased contractility at lower stimulation frequency (27, 38). It is well established that blocking BK channels in UBSM increases excitability and contractility. It is conceivable that blocking or ablating the gene for BK channels could enhance nerve-evoked contractions partly through an effect on the nerves. However, we demonstrated that UBSM strips from Slo−/− mice show increased contractility to muscarinic and purinergic agonists, consistent with a smooth-muscle loss of BK channels (38). Here, we provide evidence from immunostaining that BK channels are highly expressed in the smooth muscle, with no detectable expression in the nerves of UBSM strips (Fig. 1). Tanaka et al. (37) have shown that the BK channel opener, NS-8, can inhibit afferent nerve activity in urinary bladder; however, it was also demonstrated that this drug can have direct effects on UBSM and vascular SM as well (24). Furthermore, this class of drugs is nonspecific and may affect other ion channels (20). We cannot exclude a possible role of BK channels in afferent nerves. However, the suburothelial layer was removed, and the effects that we exhibited were due to stimulation of efferent, parasympathetic nerves. The sum of data indicates that the observed increase in nerve-evoked contractility is the result of the loss of the BK channel from the detrusor smooth muscle.

Blocking the BK channel with ibotenate or ablating its gene had differential effects on cholinergic- and purinergic-mediated force generation. Loss of BK channel function substantially increased maximal cholinergic-mediated force at higher frequencies (Figs. 2, 3, and 4A, top), whereas maximal purinergic force was lower (Figs. 2, 3, and 4A, bottom). In contrast, loss of BK channel function significantly increased force generation by both cholinergic and purinergic signaling at lower frequencies (<10 Hz). This effect was manifested in a significant leftward shift in the force-frequency relationship of both signaling pathways (Fig. 4, B and C). The sum of both neurotransmitter-mediated forces results in no change of the maximal force between WT and KO mice at frequencies >20 Hz, which is consistent with our previous finding (38). In the same report, we also showed a much lower half-maximal contraction frequency in the absence of BK channels. By separating the cholinergic and purinergic pathways, we demonstrate now that both pathways contribute significantly to the increased sensitivity to stimulation frequency due to block or loss of the BK channel.

Implications for excitation-contraction coupling in UBSM. ACh and ATP released from parasympathetic nerve varicosities trigger UBSM contraction, in part, by causing depolarization that would enhance Ca2+ entry through VDCCs (Fig. 5). The results of our present study propose that block of the BK channel or the deletion of its gene enhances contractility by increasing UBSM excitability. This, in turn, has a substantial influence on nerve-mediated contractions by increasing Ca2+ entry through VDCCs in the UBSM (Fig. 5).

Activation of UBSM P2X1R channels by nerve-mediated release of ATP would increase excitability. It is, therefore, predictable that purinergic-mediated force generation would be enhanced by the loss of BK channel function and blocked by VDCC inhibitors (14–16, 30). Muscarinic AChR stimulation also increases excitability (12, 13, 38), as well as elevates inositol 1,4,5-trisphosphate. The profound effect of loss of BK channel function on cholinergic-mediated contractions suggests an important role of this signaling pathway in UBSM membrane excitability. Consistent with this, we found that nerve-mediated contractions of UBSM strips could be essentially eliminated by inhibition of VDCCs (16). In support of this idea, smooth muscle-specific ablation of the gene encoding for the pore-forming subunit of L-type VDCCs (CaV.1.2) leads to a 10-fold reduction in AChR-agonist (carbachol)-induced contractions of UBSM and in micturition (40).

In conclusion, loss of BK channel function greatly increases the sensitivity of UBSM to nerve stimulation through both cholinergic and purinergic pathways. However, at frequencies >20 Hz, cholinergic-mediated force is enhanced and purinergic-mediated force is diminished, perhaps due to desensitization. The net effect is substantially greater force at frequencies <20 Hz and essentially unchanged force at high frequencies of stimulation. Any pathology (e.g., bladder outlet obstruction) that shifts the proportion of cholinergic to purinergic signaling would alter the frequency response of UBSM. These results underscore the important role of UBSM BK channels in nerve-mediated urinary bladder contractions.

REFERENCES


