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# The role of $Ca^{2+}$ sensitization and related pathways in developmental changes of detrusor contraction

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# The role of $Ca^{2+}$ sensitization and related pathways in developmental changes of detrusor contraction

Directed by Professor Sang Won Han

The Doctoral Dissertation
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Young Jae Im

December 2016

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This certifies that the Master's Thesis
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I received my master's degree back in 2005. Owing to my eagerness for learning and passion for the subject matter, I was able to complete the degree in a relatively short amount of time. Nevertheless, due to an unforeseen administrative problem, I had to wait three years before I could begin my doctoral program. Upon beginning my program, I quickly learned how challenging it was to work as an instructor and a lecturer, while at the same time starting my basic research program and attending to all the surgery needs. To this extent, I express my deepest admiration to all my colleagues who completed their Ph.D. program while practicing medicine.

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ABSTRACT

The role of Ca2+ sensitization and related pathways in developmental changes of detrusor contraction

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(Directed by Professor Sang Won Han)

This study was carried out to analyze the developmental changes of bladder response to cholinergic stimulation in detail, highlighting calcium sensitization (CS) and its related pathways.

Rats were divided into three groups in accordance with reported time of developmental milestones (newborn: day 1-4, youngster: day 5-14 and grown-up: day 15-28). Following cholinergic stimulation (carbachol 5 uM) the contractile response to detrusor were analyzed with respect to three phases (initial phasic, tonic and superimposed phasic contractions). Contractile responses were analyzed by dynamic and kinetic aspects. The responses were further compared in varying external calcium concentrations and in the presence of inhibitors of protein kinase C (PKC) and Rho kinase (ROCK), which are involved in CS.

The responses of newborns were contrasted to the others by the short and brisk initial phasic contractions, prominent tonic contractions and delayed participation of irregular superimposed phasic contractions. With development, phasic contractions became prominent and tonic contractions diminished. These developmental changes of phasic contractions were reproduced when exposed to increasing calcium concentrations. Application of specific inhibitors and molecular phasic

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analysis revealed that PKC was functional in tonic contractions of the newborn, whereas ROCK took over its role with development.

Within a few days of birth, rats' bladders experienced drastic changes of contractile mechanisms. These included dominance of phasic contractions from tonic contractions due to increased calcium dependence and the maturational shift of calcium sensitivity mechanism from PKC to ROCK.

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Key words: calcium sensitizing pathway, bladder, development, rats, protein kinase C, Rho kinase

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# The role of Ca2+ sensitization and related pathways in developmental changes of detrusor contraction

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(Directed by Professor Sang Won Han)

## I. INTRODUCTION

Childhood voiding dysfunction has been associated with the result of persistent immaturity of the nervous system in neonates or infants.[1] Failure of maturation is likely to delay toilet training, make recurrent urinary tract infections and allow persistence of urinary incontinence.[2,3] However, the putative switch for the maturation of bladder control is unclear, even though some relevant steps regarding the development of the bladder and central nervous system have been identified.[4,5]

Before maturation of neural voiding mechanisms, intrinsic smooth muscle contractile activity is thought to be essential for efficient bladder emptying.[6,7] Comparison of contractile properties of bladder strips between neonates and adults has revealed several characteristics in neonatal bladder contractions. These include ① larger responses to cholinergic than to purinergic stimuli,[8] ② larger spontaneous contractions at body temperature,[9] ③ absence of spontaneous contractions during the first week,[10] ④ contribution of nonmuscle myosin heavy chain,[11] and ⑤ increased calcium sensitization (CS).[12,13] The reported data are commonly associated with less calcium-mediated contractions and more calcium independent contractions in newborn bladders, but no study has fully integrated

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these findings and provided useful insight that helps to understand the overall findings.

CS is effective in keeping bladder muscle tone in the setting of limited calcium entry and is more active in newborn bladder than in adults.[13] This could be a good compensatory strategy when the availability of calcium is limited due to under-developed functional calcium influx.[12] or immature sarcoplasmic reticulum.[14] Therefore, spontaneous contractions that should rely on intact calcium availability are less seen[10] in newborn bladder as compared to adults' and this corresponds to the first few postnatal days when they cannot void spontaneously and depend voiding on perineal licking by the mother.[15] Usually, CS features decreased myosin phosphatase activity due to activation of either protein kinase C (PKC) or Rho kinase (ROCK), although which is predominant was not clarified in a previous study of newborn bladders.[13]

Interestingly, CS has also been implicated in pathologic bladder conditions.[16] In partial bladder outlet obstruction, CS was enhanced to compensate for the reduced contractility. Eventually, in case of decompensation, CS is a pathologic finding of the bladder, characterized by decreased contractile velocity due to predominance of tonic contraction and increased residual urine.[17–19] Sharing of CS as common features between neonate and pathologic bladders prompted us to consider the reason for the prominence of CS in newborns and its underlying mechanism. It is also possible that persistently elevated CS into childhood may be related to delayed maturation of bladder and childhood voiding dysfunction.

To address this speculation, I serially followed and compared the contractile patterns between newborns and other ages by modifying a previous analysis of carbachol (CCh)-mediated contractile response. Confirming increased CS in newborns, the mechanism and reason of increased CS were sought by applying

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specific inhibitors and molecular phasic analysis.

## II. MATERIALS AND METHODS

### 1. Animals and preparations

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University Health System. Timed pregnant Sprague-Dawley rats were obtained 1 week before parturition. After parturition, rats at postnatal day 0-4, day 5-14, day 15-28 were grouped and were regarded as representative groups for newborns, youngsters and grown-ups, respectively. Since the dominance of perineal-to-bladder spinal reflex pathway usually lasts postnatal 4-5 days, there is a clear need to stratify this age group into newborns. According to the report of prepubertal rat age,[20] when 3.3 days approximate 1 year of human life, a rate 2 weeks of age corresponds to 4-5 human years, which is an age when childhood voiding dysfunction is most frequent. These rats were designated as youngsters. The 4 week old rats correspond to a human age of 10-12 years of age, between childhood and adulthood. This age was the grown-up group.

Following euthanization, bladders were circumferentially cut over the dome and trigonal areas. Strips were carefully prepared with intact mucosa because of technical difficulty of peeling off mucosa in small bladder and concern of potential alteration of physiological contraction. All these processes were carried out using a stereomicroscope at ×15. Longitudinal muscle strips were dissected from the bladder anterior wall and mounted between a Grass FT-03 force transducer and a stationary clip in a water jacketed muscle organ bath containing oxygenated phosphate-buffered Tyrode solution (composition in mM: NaCl 116, KCl 5.4, $MgCl_2$ 1, glucose 5, $NaHCO_3$ 24 and $CaCl_2$ 2.0) gassed with 5% $CO_2$/95%

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$O_2$ to obtain a pH of 7.4. The solution also contained prazosin (1 μM) and α,β-methylene ATP (1 μM), which block adrenergic and purinergic receptors, respectively. Following equilibration and passive tension adjustment, the strips were first contracted with 120 mM KCl and the forces were used to normalize the force generated from CCh stimulation.

**2. Carbachol-mediated contraction of bladder: analysis by phase and comparison with development**

The optimal concentration of CCh capable of eliciting maximal contractile response at the lowest concentration was determined to be 5 μM based on the data of pilot test and previous paper of our laboratory.[21] Typically, this CCh-mediated contraction is thought to have three components (Fig. 1): an initial phasic contraction (IPC), sustained tonic contraction (STC) and superimposed phasic contractions (SPC).[21,22] Mean peak amplitudes of both IPC and STC as a marker of contractility were assessed based on computerized measurement. For the SPC, area under curve (AUC) was measured as surrogate marker of contractile activity. Moreover, I added kinetic analysis by measuring mean time to IPC peak and mean time to SPC occurrence for detailed analysis of phasic contractions. As Szell et al reported phasic contractions of newborn are likely to have fewer but individually larger spontaneous contractions than those in adults. Thus, the phasic contractions of newborn are likely to peak faster than adults that need to combine multiple small spontaneous contractions. Supporting our hypothesis, our previous report indicated faster mean time to IPC peak in neonates.[22] Each contractile activity was observed for 10 minutes.

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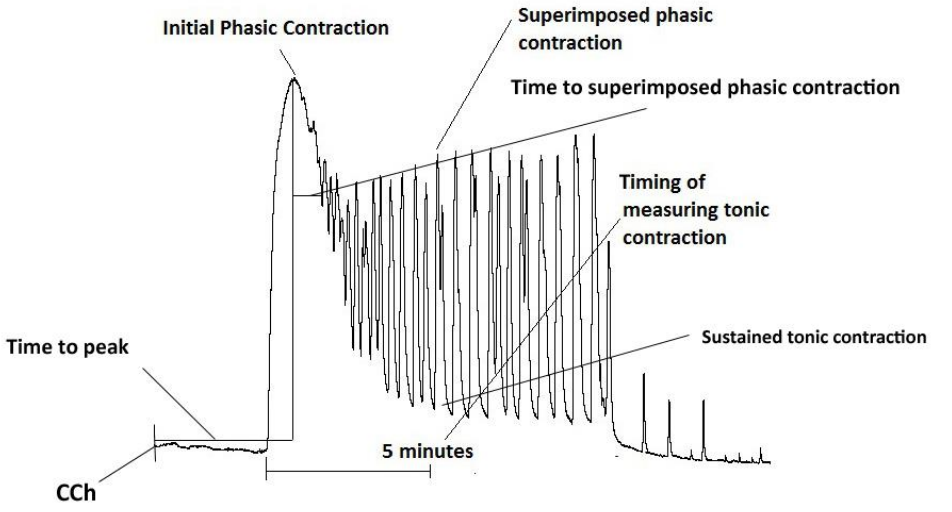


Fig. 1. Typical carbachol-mediated contractile response and method of measurement dynamic and kinetic variables.

Next, to compare the relative CS between groups, CCh-mediated contractions were re-assessed using various external calcium concentration. Extracelluar calcium was adjusted to 0, 0.5, 1, 1.5, 3 and 5 μM, realizing that all CCh-mediated contraction occurs between 1.5-3 μM $Ca^{2+}$. The three phases of phasic contraction were re-evaluated and compared between groups.

To examine the effect of two relevant pathways of CS on the CCh-mediated contraction, various doses of ROCK inhibitor Fasudil (Hydroxyfasudil; Santa Cruz Biotechnology, Shanghai, China) and PKC selective inhibitor Bis-1(Bisindolylmaleimide-I or GF 109203X; Santa Cruz Biotechnology) were applied following the administration of CCh 1 hour after resting. The effects of these inhibitors on the changes of the aforementioned variables were compared.

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### 3. Changes of phosphorylation in MYPT-1 and CPI-17 in each phase of CCh-induced contraction

An additional set of experiments were conducted to understand the changes of phosphorylation in myosin light chain (MLC), Myosin phosphatase target subunit 1 ($Thr^{696}$MYPT-1) and C-kinase potentiated Protein phosphatase-1 Inhibitor (CPI-17) in each phase of CCh-mediated contractile response. Briefly, strips were rapidly frozen in a buffer containing dry ice/acetone slurry during four timings of contraction (0, 40 seconds, 3 minutes and 5 minutes, representing basal, IPC and two times for STC to check the changing trend). Homogenized tissues were immersed in 50 ul 1x RIPA lysis buffer and then centrifuged and assayed for amount of protein in supernatant. It was washed and suspended in urea-glycerol buffer for 8-12% SDS-PAGE to separate phosphorylated and nonphosphorylated form based on mass and charge differences. Following transfer to a polyvinylidene fluoride membrane and blocking for 1 hour, the membrane was incubated with rabbit anti-myosin light chain 2 antibody (1:1000) and its phosphorylated form (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membrane was briefly rinsed with Tris buffered saline-Tween (TBS-T) buffer and incubated with secondary antibody conjugated with horseradish peroxidase (1:3000) for 60 minutes. Bands were detected by enhanced chemilluminescence. Phosphorylation levels were calculated by densitometric analysis of phosphorylated forms divided by total forms. Similar processes were conducted for assaying phosphorylation in CPI-17 and MYPT-1, and their phosphorylated forms, except for the use of different concentration of SDS-PAGE gel electrophoresis (8% for MYPT-1).

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### 4. Statistics

Data are shown as the mean ± SD and analyzed using GraphPad Prism Version 6 (San Diego, CA, USA) with one-way analysis of variance (ANOVA) followed by the Tukey post-hoc correction. When data were not distributed normally or heterogeneity of variance was identified, nonparametric Kruskal-Wallis analysis was used instead.

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III. RESULTS

### 1. Changing patterns of typical CCh-induced contractile response

The left panel in Fig. 2 shows the typical response to CCh among the three age groups. Youngsters and grown-ups showed the three typical phasic responses, definite IPC followed by the relatively stable STC and SPC. With development, the amplitudes of STC and SPC diminished and the frequencies of SPC became higher, explaining the AUC increase. On the other hand, responses in the newborns featured brief and steep IPC directly attached to prolonged STC at the peak and delayed, irregular SPC that decreased as STC gradually descended. The responses of the newborns were characterized by obviously lower IPC, higher STC and smaller AUC of SPC (Figs. 2A-C). The mean time to peak of the IPC was much shorter and the time to SPC was significantly prolonged compared to the others (Figs. 2D and 2E). Taken together, the contractile responses tended towards higher IPC, lower STC and more frequent SPC with age.

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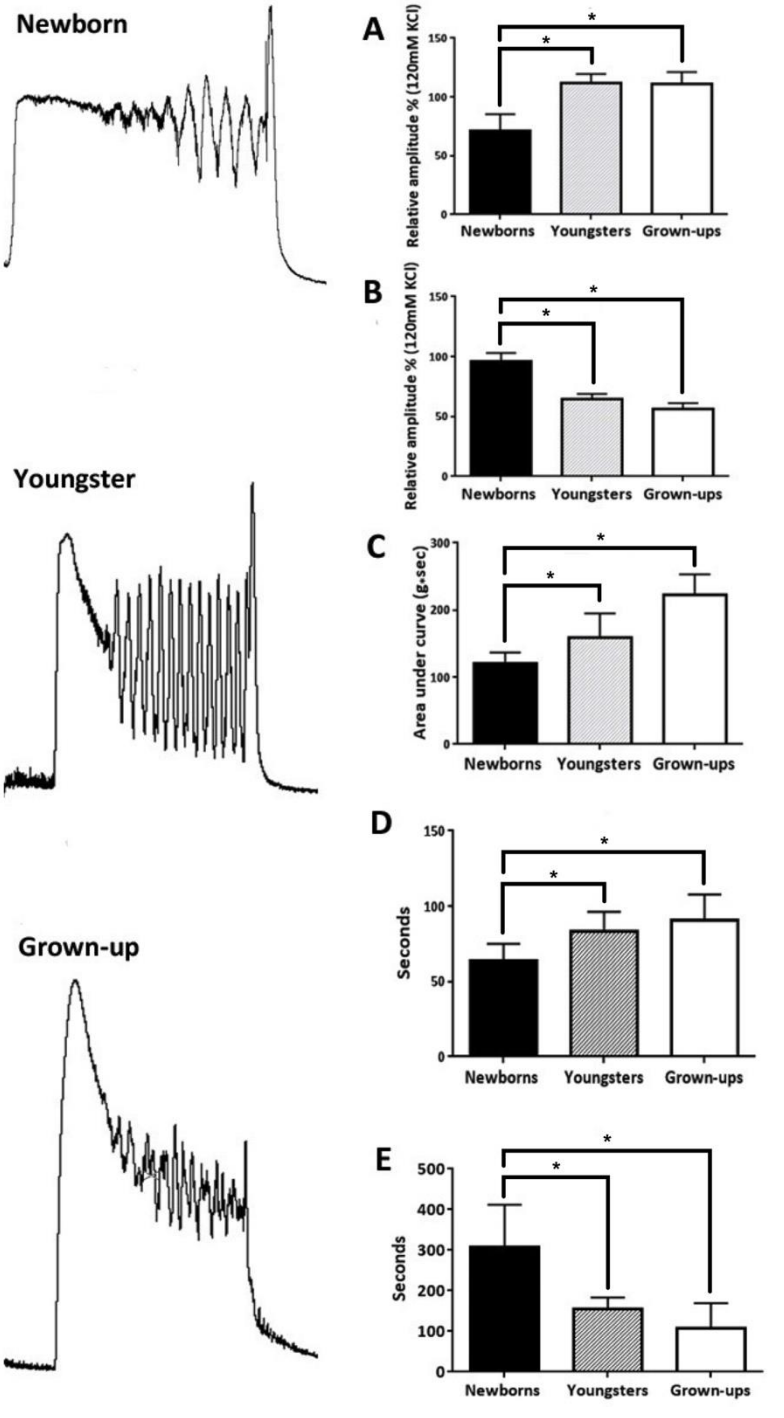

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Fig. 2. Left panel, original traces of carbachol-mediated contractile response for newborns, youngsters and grown-ups.

A. Comparison of mean amplitude of initial phasic contractions
B. Comparison of mean amplitude of sustained tonic contractions
C. Comparison of mean area under curve of superimposed phasic contractions
D. Comparison of mean time to peak of initial phasic concentrations
E. Comparison of mean time to superimposed phasic contractions

Newborns (N=4), youngsters (N=4) and grown-ups (N=4) are depicted in black, shaded and open bars, respectively and * denotes statistical significance ($p<0.05$).

## 2. Comparison of responses to CCh in changing external calcium concentration

Newborns showed noticeable contraction following administration of 1.5 mM calcium, whereas such contractile response was not seen until the addition of 3 mM calcium in older groups (Fig. 3). With increasing concentration of calcium, the amplitude of IPC, STC and AUC of SPC showed proportional increases. Regarding kinetics, decreasing tendency of the mean time to peak of IPC and time to SPC was observed, especially in the newborns (Fig. 3).

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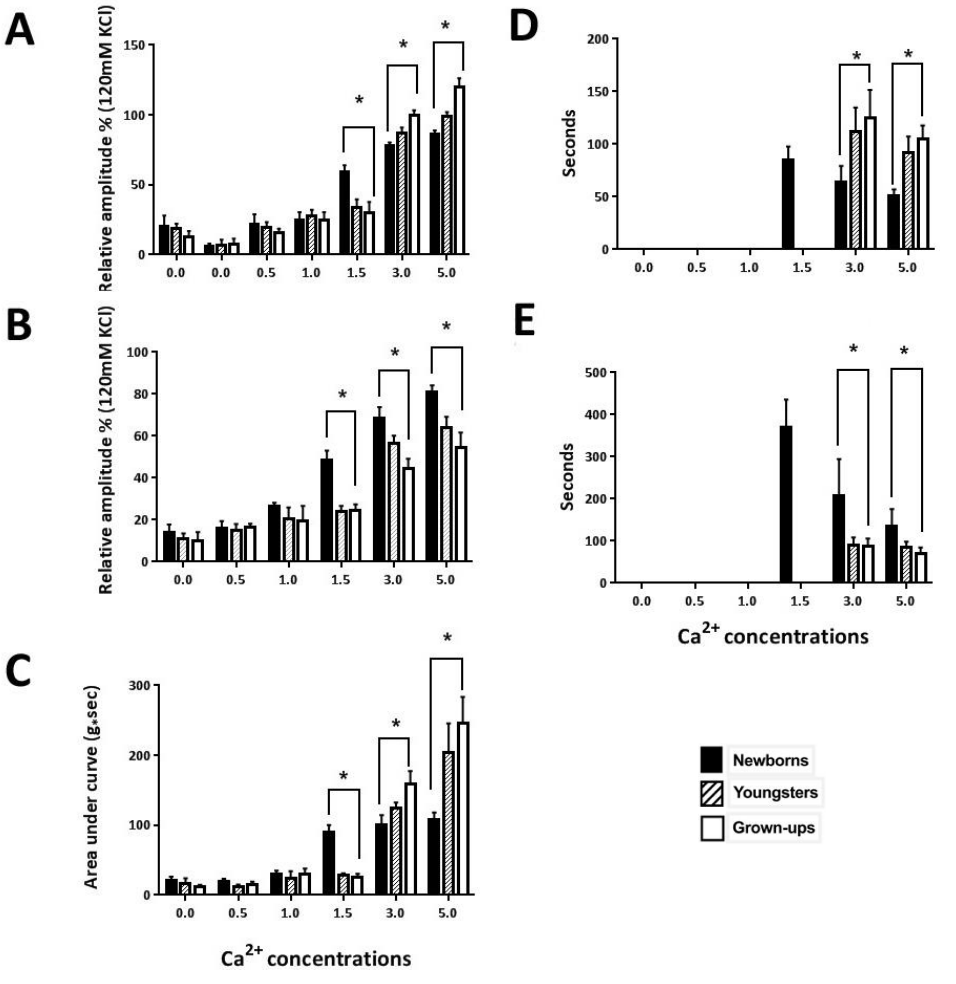


Fig. 3. Carbachol-mediated contractile responses to increasing concentration of external calcium administration.

A. Comparison of mean amplitude of initial phasic contractions
B. Comparison of mean amplitude of sustained tonic contractions
C. Comparison of mean area under curve of superimposed phasic contractions
D. Comparison of mean time to peak of initial phasic concentrations
E. Comparison of mean time to superimposed phasic contractions

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Newborns (N=4), youngsters (N=4) and grown-ups (N=4) are depicted in black, shaded and open bars, respectively and * denotes statistical significance ($p<0.05$).

### 3. Effects of potential inhibitors of calcium sensitization on CCh-induced contraction

Effect of various concentrations of either Bis-1 or Fasudil on CCh-mediated bladder contraction is shown in Fig. 4. Treatment with Bis-1 resulted in significant reduction of all contractile responses of the newborns in a clear dose-responsive manner. Conversely, no significant inhibitory action of Bis-1 was seen in contractile responses of the other groups. Regarding kinetic variables, the newborn group showed the significant reduction of mean time to IPC peak and mean time to SPC at 0.3 and 3 uM ofBis-1 compared to baseline. In the other groups, treatment of Fasudil resulted in reduction of contractile response at all phases with a clear dose response relationship, however this drug had no effect on kinetic variables.

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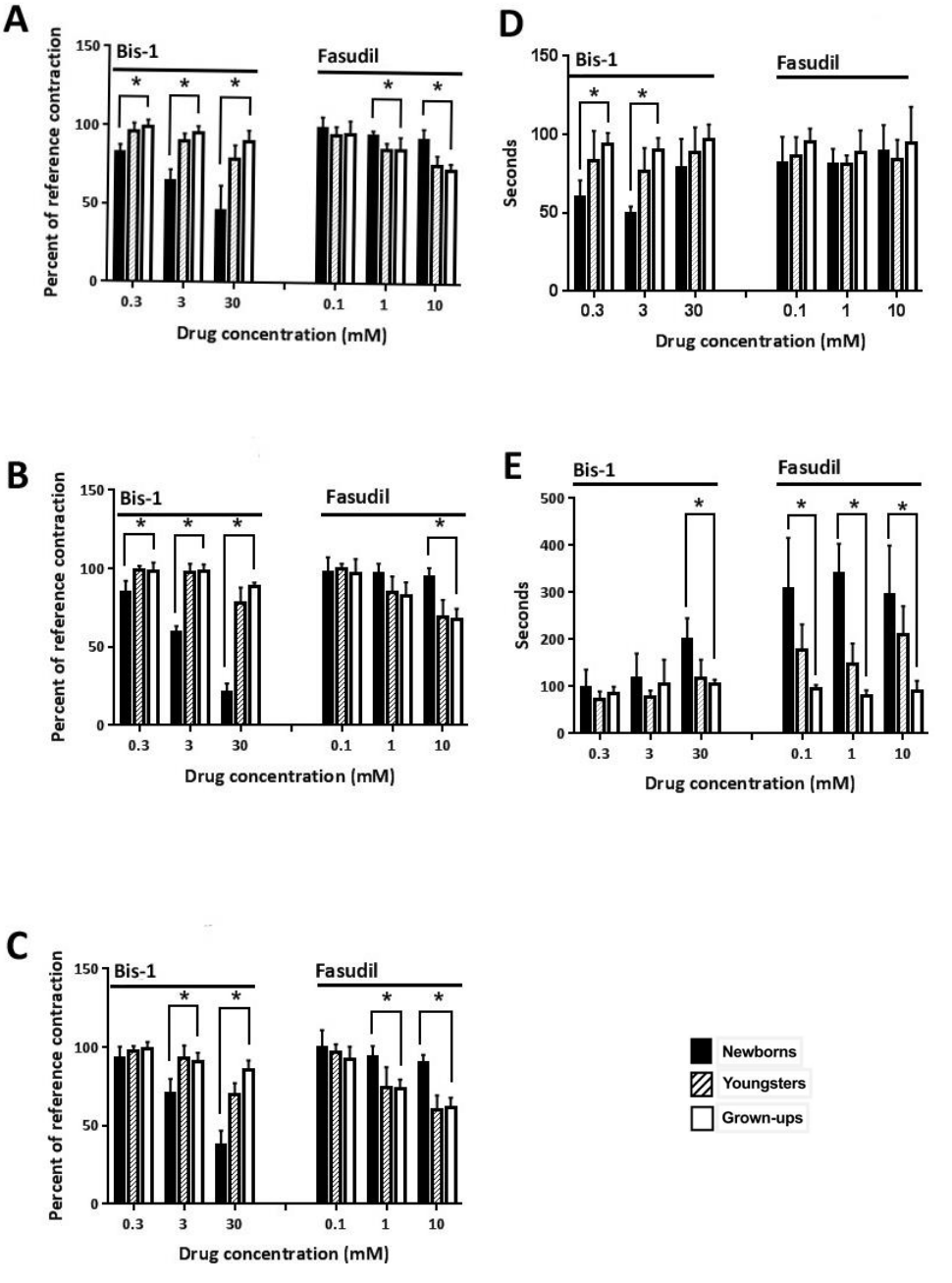

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Fig. 4. Carbachol-mediated contractile responses to varying concentrations of Bisindolylmaleimide-I (Bis-1) and Hydroxyfasudil (Fasudil).

A. Comparison of mean amplitude of initial phasic contractions
B. Comparison of mean amplitude of sustained tonic contractions
C. Comparison of mean area under curve of superimposed phasic contractions
D. Comparison of mean time to peak of initial phasic concentrations
E. Comparison of mean time to superimposed phasic contractions

Newborns (N=4), youngsters (N=4) and grown-ups (N=4) are depicted in black, shaded and open bars, respectively and * denotes statistical significance ($p<0.05$).

### 4. Phosphorylation of MLC, CPI-17 and MYPT-1 during each phase of CCh-mediated contraction

In all tested groups, MLC phosphorylation was highest at 40 seconds. However, only the newborn group maintained a significant elevation of MLC phosphorylation until 5 minutes. This was associated with elevated phosphorylation of CPI-17 up to 5 minutes in the newborns. No significant elevation of phosphorylated CPI-17 was seen in the others. On the contrary, the latter showed significantly elevated phosphorylated MYPT-1 in both 3 and 5 minutes of contractions with no significantly elevated phosphoMYPT-1 in newborns.

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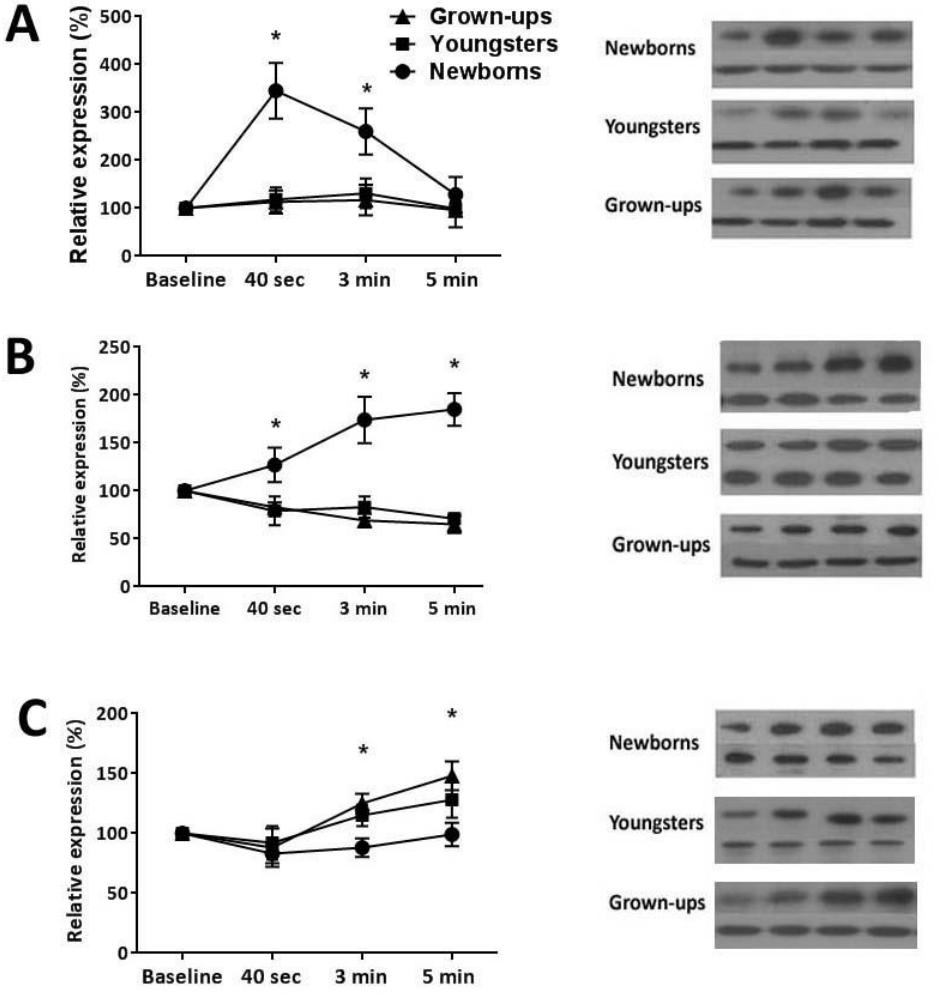


Fig. 5. Molecular assay regarding (A) phosphorylation of myosin light chain (MLC), (B) C-kinase dependent phosphatase inhibitor of 17 kD (CPI-17) and (C) myosin phosphatase targeting subunit-1 (MYPT-1) in specific timings representative of phasic and tonic contractions.

Trend of densitometry is depicted in left and representive western blots are paralleled in right side. * denotes statistical significance ($p<0.05$).

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## IV. DISCUSSION

The IPC of newborns was discriminated from the older groups by an asymmetric shape featuring a small and rapid upshoot followed by gradual descent and delayed occurrence of SPC. It is known that IPC is associated with spontaneous contractions due to opening of calcium channels and these spontaneous contractions can be elicited by accumulation and spread of pacemaker activity which is responsive to cholinergic stimuli.[4,6,8,9] Spontaneous contractions in newborn bladder has been described to have large amplitudes and infrequent occurrence owing to small participating number of pacemaker activity and less functional gap junctions. Thus, the increase in IPC of newborns is like to be small and rapid due to the small number of pacemakers.

In contrast to the rapid increase, a more gradual decrease was evident compared to older age groups resulting in asymmetric IPCs. This gradual descent could be caused by early incorporation of tonic contractions. This assumption was consistent with the gradual pressure changes of newborns and consistent with the previous findings of CS dominance of newborn bladders.[13,16] Although it will be discussed later, the highest phosphorylated CPI-17 at 40 seconds also corroborates this claim. The increased CS in the newborn took on the role from the downhill instead of weak IPC.

The AUC of SPC increased with development due to increasing frequencies. This showed similar feature with developmental changes of spontaneous contractions, becoming chaotic with increasing frequencies. This suggests the possible association between SPC and spontaneous contractions. This change may fit for silencing of the bladder during the storage phase, increasing capacity and recruiting multiple foci of spontaneous contractions to produce sufficient power for emptying during voiding phase.[23]

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The aforementioned results clearly indicate that developmental changes of bladder involve an increasing role of phasic contractions from prevailing tonic contractions.

The newborn bladder showed typical contractile response from 1.5 mM of calcium, whereas the other groups showed this from 3 mM, supporting the previous results of increased CS in newborns.[12,13] Moreover, one salient finding was that increasing extracelluar calcium concentrations enhanced phasic contractions, nearly reproducing developmental contractile responses. Also, the mean time to IPC peak and SPC was also significantly reduced supporting above-mentioned claim. This indicates that increased utility of calcium is associated with the bladder development. On the contrary, tonic contractions displayed an opposite direction to developmental changes, showing enhanced responses to increasing calcium concentrations. This suggests that even a neonate bladder could respond to increased calcium but the paucity of calcium was the reason of increased CS and prevailing tonic contractions in this ages.[14]

Following confirmation of the predominance of CS in bladders of newborns, I investigated which mechanism was responsible for the CS in newborn bladder contractions. In newborns, significant inhibitory action of Bis-1 and significantly elevated expression of phosphorylated CPI-17 during whole phase were observed, suggesting functionality of PKC in whole contractile process. This appeared to delay the time to SPC in newborn given the inhibitory role of PKC on spontaneous contractions,[24,25] But this was disappeared soon after the newborn period. This transient nature of PKC action in newborn has also been demonstrated.[24]

The interpretation of kinetic data following treatment of PKC inhibitors seems more complex. Since PKC is reported to enhance large conductance potassium

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channel, inhibiting spontaneous contractions, administration of Bis-1 is likely to decrease mean time to IPC peak and SPC. However, comparing Fig. 4 with the original Fig. 2 revealed no actual change in mean time to IPC peak. Only mean time to SPC was more decreased in newborn. This requires more investigation and may indicate complex action of PKC in bladder.[25]

In contrast to the case of PKC, the newborn showed no notable evidence for ROCK activation. The activation of this pathway was seen when rats aged, showing transition from PKC to ROCK with development. Interestingly, the assay for phosphorylated MYPT-1 revealed activation 3 and 5 minutes after the contractile responses, while administration of Fasudil affected all responses except the newborn, not limited on tonic contractions. The reason of this discrepancy between molecular expression and force generation is unclear. Possibly, phosphorylation of $^{696}$Thr-MYPT-1 may not be so sensitive to detect the changes during phasic contractions. The data support a previous suggestion of a functional role of another isoform of phosphorylated $^{850}$Thr-MYPT-1 in rabbit bladder.[26] Examining both isoforms might lead to synchronization of both tests.

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## V. CONCLUSION

In this study, the CCh-mediated contractile responses among age groups were compared to reveal the developmental differences and to speculate the possible mechanism. The major findings were a notable difference of contractile response between newborn (< 5 days) and the older groups, reproducible developmental changes of contractile responses by increasing calcium concentrations, a crucial role of PKC in contractile response in newborns, and maturational shift to Rho kinase after newborn period. These confirmed the previous data,[21,22.24,27] and provides additional insight that may help to understand mechanism regarding development of bladder.

Based on results of this study, prolonged transition of CS into childhood may play a role in voiding dysfunction, especially overactive bladder. Although elevated CS due to ROCK activation has been associated with several pathologic conditions in adult, PKC rather than ROCK is considered as the potential candidate pathway in pediatric voiding dysfunction.

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ABSTRACT(IN KOREAN)

# 방광근육수축의 발달과정에서 칼슘민감성수축의 역할 및 관련 분자경로

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소아의 배뇨과정의 발달은 아직 충분히 알려져 있지 않지만 방광근육수축의 발달과 밀접한 관련이 있다. 방광수축은 평활근내의 myosin light chain의 인산화로 결정되며 이는 세포내 칼슘농도에 의존하는 기전과 세포내의 칼슘농도의 변화가 거의 없이도 지속적 방광수축을 가능하게 하는 소위 칼슘민감성수축으로 구분된다. 본 연구는 현재까지 연구가 잘 이루어지지 않은 칼슘민감성수축과 이와 관련된 분자경로에 주목하여, 방광근육수축이 연령에 따라 어떻게 변화하는 지를 분석하였다.

백서를 발달시기별로 세 군으로 나누어 (신생아기: 1-4일, 유아기: 5-14일, 청소년기: 15-28일) 콜린성자극 (carbachol 5μM)에 대한 방광근육의 수축을 세가지 단계별 (초기 상동성 수축, 긴장성 수축, 적재성 상동성 수축)로 분석하였다. 실험은 방광근육절편을 이용하였으며, 다양한 세포외 칼슘농도 및 칼슘민감성수축에 관여하는 분자경로인 protein kinase C (PKC)와 Rho-kinase (ROCK)의

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길항제의 유무에 따른 수축반응의 변화를 비교 분석하였다.

신생아 백서의 방광근육수축의 특징은 짧고 빠른 초기 상동성 수축, 뚜렷한 긴장성 수축 및 지연되어 나타나는 불규칙적인 적재성 상동성 수축이었다. 이러한 신생아기의 특징은 연령이 증가함에 따라, 상동성 수축이 더 뚜렷해지고 긴장성 수축은 감소하는 경향을 나타냈다. 세포외 칼슘농도를 변화시키며 분석한 결과 신생아기의 백서에서는 상대적으로 낮은 세포외 칼슘농도에서도 방광근육수축이 유발되어 다른 연령군에 비해 칼슘민간성수축이 더 두드러지는 것을 확인할 수 있었다. 칼슘민감성수축과 연관된 분자경로의 분석결과 신생아기의 긴장성 수축에는 PKC가 주요 역할을 하며, 연령이 증가함에 따라 ROCK가 그 역할을 대체하는 것으로 나타났다.

생 후 수일안에, 백서의 방광수축기전은 다음과 같은 급격한 변화를 보였다. 긴장성 수축 우위에서 칼슘의존성 증가에 따른 상동성 수축 우위로 바뀌었으며, 칼슘민감성 기전은 PKC에서 ROCK로 성숙에 따른 변화를 나타냈다.

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핵심 되는 말: 칼슘민감성 경로, 방광, 발달, 콜린성, 백서