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Putative K^+ channel in *Schizosaccharomyces pombe* is regulated by H^+, K^+ and cAMP at transcriptional level

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Summary. Living cells control their electrical responsiveness by regulating the quality and quantity of channels expressed in the plasma membrane. Regulation of transcription of the voltage-gated ion channels is an important part of the molecular basis of cell energization. However, the factors which control the expression of channels are not well understood. We studied the effect on the transcription of the voltage-gated K^+ channel in the yeast *Schizosaccharomyces pombe* of cations, pH, and therapeutic spasmolytic and hypotensive agents with different mechanisms of action, including accumulation of intracellular cAMP. A highly specific 122 bp domain of the K^+ channel between S5 and H5 with a 55% homology with Dros shab and mbk3 was amplified by nested PCR from chromosomal DNA *S. pombe*. Northern blot revealed a 1.8 kb transcript. mRNA dot-blot and RNase-protected analysis revealed factors altering the K^+ channel transcription.

Keywords: Amino acids – Potassium channel – Yeast – Transcription regulation – Spasmolytic and hypotensive drugs

Introduction

Ion fluxes across cell membranes mediate a variety of biological processes that are essential for the viability of cells. These fluxes occur in processes as complex as muscle contraction, exocrine cell secretion and nerve excitability. Passive fluxes across membranes are mediated by membrane channels which can be divided into several families according to their ion selectivity. The fundamental feature of these ion channel families is that there is more structural similarity among members of a given ion channel family of different species than among ion channels of a given species belonging to different families (Ranganathan, 1994). Numerous studies have shown that the voltage-gated K^+ channels play fundamental basic role in the cell responsiveness to intracellular and extracellular signals. The mechanism of action of these chan-

nels is generally well known. However, the mechanisms of regulation of expression of the voltage-gated K^+ channels are less clear. The low levels of gene expression in mammalian cells make it difficult to study the regulation of the channel protein expression at the pre- and post-translational levels (Mori et al., 1993). The successful cloning and expression of K^+ channels from the Shaker locus of *Drosophila* and the mammalian homologues from rodents have made it possible to study the structure-function relationship of these channels (Mori et al., 1993). However, the specificity of the K^+ channels as well as their similarity with other channels call for the determination of their structural organization, transcriptional regulation, and tissue-specific expression.

In the unicellular eukaryotic organisms, the ionic fluxes play a key role in various activities such as cell volume regulation and cell motility. The structure of K^+ channels among these and the multicellular organisms is highly conserved. It is not clear whether this is true also for their functions. The fact that the cells of unicellular organisms possess all the functions of interest makes these organisms suitable for studying both the similarity of mechanisms of regulation of the evolutionarily conserved voltage-gated K^+ channels and the divergence in their regulation due to cell differentiation.

We studied the mechanisms of the K^+ channel regulation using *S. pombe* as a model unicellular eukaryotic organism. We characterized several factors affecting expression of the K^+ channel at the transcriptional level under different cationic growth conditions. In addition, we studied the effect of several drugs which are commonly used in therapeutic spasmolytic and hypotensive therapy. One of the compounds, papaverinum hydrochloride, is an inhibitor of phosphodiesterase and thus significantly increases the intracellular level of cAMP (Mashkovskyi, 1977). We have reported earlier on the electrophysiological properties of the K^+ channel in *S. pombe* as determined by the patch-clamp technique (Vacata et al., 1993): its conductivity of 153 pS is considerably higher than 50 pS of their equivalents in *Saccharomyces cerevisiae* (Gustin et al., 1988). In spite of the high sequence homology among the members of the voltage-gated K^+ channel family we therefore proposed a divergence among the *S. pombe* channels within the sequence of S4–S6 segments which were shown to be responsible for the channel's selective conductivity (review by Ranganathan, 1994).

Materials and methods

Chemicals

Electrophoretic agarose was purchased from Sigma; restriction endonucleases, PNK, calf intestine alkaline phosphatase (CIAP), and PCR kit from Boehringer Mannheim; SURE CLONE ligation kit from Pharmacia; (α-^{32}P)-dATP, (α-^{32}P)-dCTP, (γ-^{32}P)-dATP, nylon membranes Hybond, and DNA labeling kit from Amersham; oligonucleotides were synthesized using a standard method with HPLC purification in MWB; barbituric acid was purchased from Serva (0.01% in growth medium); papaverinum hydrochloride (2% solution, pH 4.5), dibazolum hydrochloride (1% solution, pH 6.0) from Moldavanopharma (0.01% in growth medium). All other chemicals were purchased from common commercial sources.

Media

Full medium: 3% glucose, 0.5% yeast extract, pH 4.5; *Minimal medium*: 0.2 M glucose, 11.4 mM asparagine, vitamins per 1l medium: 0.1 mg Ca-panthenate, 1 mg nicotinic acid, 1 mg inositol, 1 μg biotin; macroelements: 7 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM NaCl, 1 mM $CaCl_2$, 40 mM $(NH_4)_2SO_4$; trace elements: 8 μM H_3BO_3, 0.2 μM $CuSO_4$, 0.6 μM KI, 0.7 μM $FeCl_3$, 2 μM $MnSo_4$, 0.8 μM H_2MoO_4, 1 μM $ZnSO_4$ (Gutz et al., 1974).

Strains

Schizosaccharomyces pombe, 972 h^- (NCYC 1827 h^-) – wild type, *Escherichia coli* DH 5α were from ATCC.

Cultivation of S. pombe

In full and/or minimal medium cultivation at pH 4.0, 4.5, 5.0. The cells were grown to saturation in full and minimal media on a rotary shaker at 28°C, harvested by centrifugation at 2,000 g for 15 min, rinsed with 0.9% saline and frozen at −70°C.

PCR amplification of a fragment of a voltage-gated K^+ channel by nested PCR

PCR I

A downstream 20-mer oligonucleotide (primer I) was derived from a degenerative amino acid sequence R(I,V)(L,F,M) RI(F,L)K of the S4 domain deduced from published alignment (Anderson et al., 1992). The degenerative sequence of primer I is homologous to the coding strand 5′MGVATNWTBMGNATHYTYAA3′. The upstream 17-mer oligonucleotide (primer II) was derived from the amino acid sequence TVGYGD of the H-domain. Primer II has a degenerative sequence 5′TCNCCRTANCCNACNGT 3′. The oligonucleotides were designed taking into account the codon preference of the yeast. 100 ng of chromosomal DNA isolated by a standard method (Sambrook et al., 1989) were used as a template for PCR I. DNA template was preheated for 100 min at 95°C. 30 cycles (1 min 94°C, 1.5 min 48°C, 2 min 65°C) of PCR I were performed in the presence of DNA template, 500 pM of each primer (I and II), 200 μM dNTPs, 10 mM Tris-HCl, pH 7.4 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 2.5 U Taq polymerase in a total volume of 50 μl, followed by a final extension for 10 min at 65°C.

PCR II

The downstream 20-mer oligonucleotide was derived from a degenerative amino acid sequence AE(K,R,A)(D,I)(E,S,D,G,Q)(D,P)(D,N) of the S5 domain. The degenerative sequence 5′GCAGARRVNRWWVRNSMDR 3′ of primer III is homologous to the coding strand. Similarly to PCR I, primer II was used as the upstream one. An aliquot of total PCR I products (100 ng) was used as template in PCR II, which was performed under the same conditions as PCR I but with primers II and III. Purified major DNA products from both PCR I and II were cloned at Sma I site of pUC 18 in *E. coli* DH 5α.

Sequencing of DNA fragments

was performed in the Max-Planck Institute in Cologne, Germany (laboratory of Prof. K. Palme).

Northern and mRNA dot-blot analysis

mRNA was isolated from 200 mg of each set of cultivated *S. pombe* cells by Micro-Fast-Track kit (Invitrogen) using procedure recommended by the manufacturer, and stored in ethanol at −70°C until further use. The spectrophotometrically determined concentration of purified mRNA in water was about 5–10 μg in each sample. Standard Northern-blot analysis was performed with 1 μg of mRNA (Sambrook et al., 1989) using 2×10^6 μCi of (α-^{32}P)-dCTP-labeled K^+ channel specific probe, amplified by nested PCR. The membrane was autoradiographed for one week at −70°C. For dot blotting, 100 ng aliquots of mRNA were denatured using a standard procedure (Sambrook et al., 1989) and applied to nylon membranes using vacuum blotting apparatus (Bio-Rad). Hybridization was carried out with the same probe as in the Northern-blot analysis under standard conditions. Washed membranes were autoradiographed for 72 h. The signals on the exposed film were quantified by densitometric scanning using LKB 2202 ultrascan laser densitometer.

RNase protection analysis

was performed using a modified procedure of Matsubara et al. (1991). A riboprobe was derived from a 122 bp fragment cloned in pUC 18. Recombinant plasmid pUKSp63.1 was linearized by EcoRI, and isolated and purified from 1% agarose electrophoretic gel. Riboprobe of approx. 130 bp was synthesized as a noncoding strand from PCR primer H5 on the coding strand of linearized pUKcSp63.1 by Taq polymerase using labeled dCTP and dATP. The product of polymerization was denatured from longer nonlabeled strand and labeled ssDNA of approx. 130 bases was isolated from 2% agarose gel. 2×10^5 cpm heat-denatured riboprobe (~10 ng) was overnight hybridized in excess with 1 μg of each mRNA sample. After hybridization, mRNA was digested with RNase A (10 μg/ml) at 37°C for 15 min prior to electrophoresis on 2% agarose gel. As a control of the length of the transcript-protected fragment we used the specific 122 bp probe amplified with PCR using (α-^{32}P)-dCTP. The gel was autoradiographed in Saran wrap at −70°C. The signals were quantified the same was as in the dot-blot analysis by densitometric scanning in the LKB 2202 Ultrascan Laser Densitometer.

Comparison of sequences

Identification of the determined DNA and the corresponding amino acid sequences was made by a comparison with databases using Internet.

Results

Preparation of a highly specific probe for further determination of K^+ channel at transcriptional level from *S. pombe* and its structural analysis is shown in Figs. 1–4. Northern blot analysis and K^+ channel transcriptional quantification at different growth conditions is shown in Figs. 5–6.

Discussion

We used nested PCR to prepare a specific probe for the Northern blot hybridization and the RNase protection analysis. A 122 bp fragment was cloned and sequenced from chromosomal DNA of *S. pombe*. The correspond-

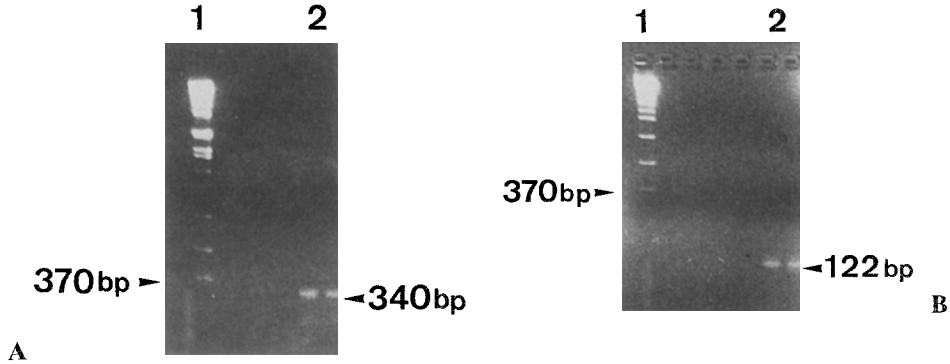

Fig. 1. Analysis of PCR I (**A**) and PCR II (**B**) products in 2% agarose gel. Lane *1*, DNA standard No VII (Boehringer); lane *2*, total products of PCR

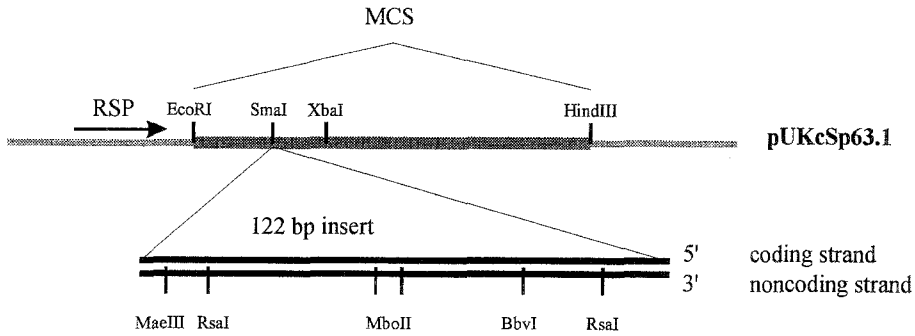

Fig. 2. Restriction map of recombinant plasmid pUKcSp63.1. MCS – multiple cloning site of pUC 18; RSP – reverse sequencing primer

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5'
   1     GCA GAG GGC GAT GAG GCT GAG TCT AGT ACA GTA
         --------primer III------->
   34    AGG CAG CGA GGA GGG AAA AGA AGA TTT CCC ATA

   67    TGT TGG GAA GAG AGA TTT GAG AGA GGG AGA GAA

   100   GTA CGG ACG GTC GGT TAC GGA GA   122 bp
               <------primer II------
3'
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Fig. 3. The determined nucleotide sequence of target 122 bp product of nested PCR

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                **.***.*. .      . .*.   *  .  *     ******
S. pombe        AEGDEAESSTVRQRGGKRRFPICWEERFERGREVRTVGYGD
1               AEKDEKD           TKFVSIPEAFWWAGITMTTVGYGD
2               AEKDEDD           TKFKSIPASFWWATITMTTVGYGD
3               AEKDEDD           TKFKSIPASFWWATITMTTVGYGD
4               AERIGAQPNDPSASE HTHFKNIPIGFWWAVVTMTTLGYGD
5               AERIQPNP          HNDFNSIPLGLWWALVTMTTVGYGD
6               AEADDPSSG            FNSIPDAFWWAVVTMTTVGYGD
7               AEAGSENSF            FKSIPDAFWWAVVTMTTVGYGD
                S5                                    H5
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Fig. 4. Alignment of determined amino acid sequence of S5-H5 segment of voltage-gated K^+ channel from *S. pombe* with *1.* Dros shab, *2.* drkl, *3.* Mouse shab, *4.* ngk2, *5.* shaw, 6. mbk3 and *7.* ShA where K^+ channels are from *Drosophila* (Dros shab; shaw; ShA), mouse (Mouse shab, ngk2, mbk3) and rat (drk1) (Anderson et al. 1992).* – identical residues; · – similar residues

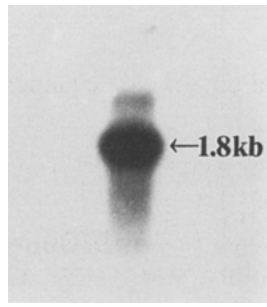

Fig. 5. Northern-blot analysis of K^+ channel transcription in *S. pombe*

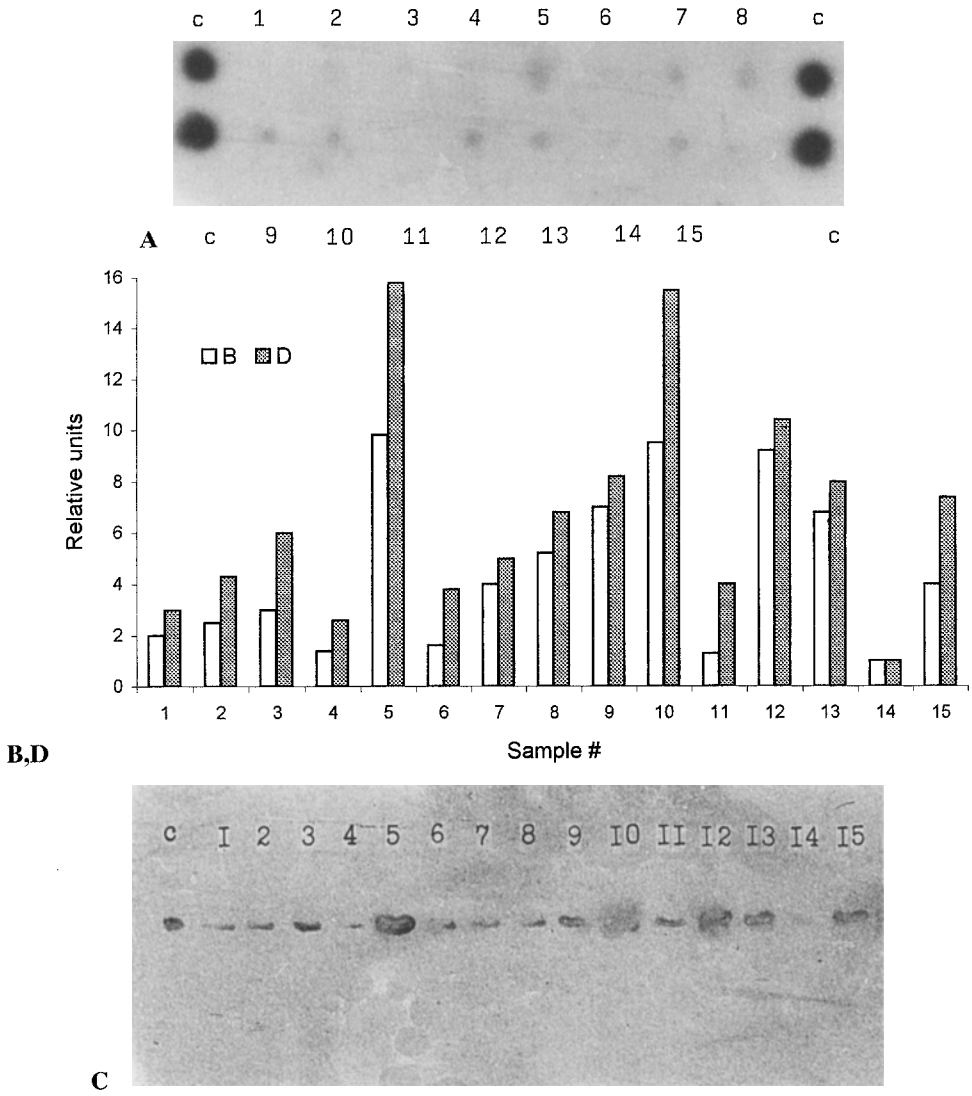

Fig. 6. mRNA dot-blot (**A**) and RNase-protection (**C**) analysis of the regulation of K^+ channel transcription in *S. pombe*. Dot-blot quantification (**B**) and RNase-protection quantification (**D**) analysis expressed in relative units in comparison with the minimal transcription level taken as 1. Numbered samples correspond to the following growth conditions: *1–3*, full medium with pH 4.0, 4.5, 5.0 respectively; *4–6*, full medium, pH 4.5, supplemented with dibazolum, papaverinum and barbituric acid respectively; *7–9*, minimal medium with pH 4.0, 4.5, 5.0 respectively; *10* and *15*, minimal medium, pH 4.5, supplemented with papaverinum; *11–13*, minimal medium, pH 4.5, free of Mg^{2+}, Na^+, Ca^{2+} respectively; *14–15*, minimal medium, pH 4.5, with 0.6 mM K^+. K in **C** is a labeled EcoRI/HindIII digested insert of pUKcSp63.1 as a control of length of transcript-protected fragment. *c* – labeled probe as a control

ing amino acid sequence of the amplified fragment contains S5 and highly specific H5 domains of the K^+ channel with a 55% homology to Dros shab (Butler et al., 1989) and mbk3 (Chandy et al., 1990). The Northern blot revealed a 1.8kb corresponding transcript. The differences at the transcriptional level were quantified by densitometric scanning of the mRNA dot-blots. The results were confirmed by the RNase-protection analysis experiments; they showed differences between the K^+ channel transcription in minimal and full media which were not significantly affected by pH between 4 and 5. It also revealed downregulation of transcription at low concentrations of K^+ and Mg^{2+}. In contrast, low concentrations of Ca^{2+} and Na^+ slightly increased the level of expression of the K^+ channel. The inhibition of phosphodiesterase accompanied by cAMP accumulation significantly increased the expression of the K^+ channels; this effect is in agreement with the positive response of the transcription to cAMP as observed in primary cardiac cells (Mori et al., 1993).

The genetic characterization of K^+ transport in *S. cerevisiae* (Gaber et al., 1988) points at a functional independence of the high/low affinity uptake transport systems of the passive K^+ transport mediated by channels (Gaber et al., 1988). On the other hand, net K^+ efflux was observed from both the trkl and the wild type cells apparently due to the K^+ gradient. Moreover, binding of nucleotides to the putative nucleotide-binding domain in Trkl may play only a regulatory role, and Trkl might mediate passive transport by acting as a K^+ channel. The functional relationship between the active and the passive K^+ transport systems and/or the regulatory factors thus remains unclear.

The viability of the trklΔtrk2Δ cells reveals an existence of a 3d K^+ transporter: under standard conditions this low pH-sensitive system is not involved in the bulk K^+ accumulation in cells (Ko and Gaber, 1991). This transporter in trklΔtrk2Δ cells may mediate K^+ transport primarily to regulate pH homeostasis or the electrical potential. Apart from the possible K^+/H^+ antiport this protein could also serve as a K^+ channel. High proton concentrations could either close the channel or inhibit K^+ uptake by competitive inhibition (Ko and Gaber, 1991).

The current study deals with the determination of factors regulating the expression of voltage-gated K^+ channels in *S. pombe* at a transcription level. The protein and the gene encoding the K^+ channel in *S. pombe* and *S. cerevisiae* have not yet been characterized. Because of the high conservation of this family of polytopic membrane proteins we amplified the 122bp DNA fragment encoding the S5-H5 segment of the voltage-gated K^+ channel (Figs. 1, 2 and 3). The corresponding amino acid sequence reveals a 55% homology to Dros shab and mbk3 proteins (Anderson et al., 1992) (Fig. 4). This highly specific probe was used in the Northern blot analysis, mRNA dot-blot and RNase-protection quantification analysis. Fig. 5 shows a 1.8kb transcript corresponding to the putative K^+ channel. The pH-dependent positive regulation of the K^+ channel transcription in full and minimal growth media is shown in Fig. 6. The transcription of the K^+ channel is downregulated by the electrical membrane potential in cells growing in 0.6 mM K^+ in minimal medium. This finding is in agreement with the effect of positive transcription regulation by

depolarization of membrane with 40mM KI which was observed in a primary culture of neonatal rat atrial cardiocytes (Mori et al., 1993). It is also in agreement with the observation that yeast cells grown in millimolar K^+ and subsequently transferred to K^+-free medium lose K^+ till the net K^+ uptake switches on (Ramos and Rodriguez-Navarro, 1986).

Because of the high conductivity and the low ion selectivity of the 153pS K^+ channel in *S. pombe* (Vacata et al., 1993), the absence of Na^+ in medium only slightly alters transcription of this K^+ channel. This notion ought to be confirmed in further electrophysiological experiments. Shortage of Mg^{2+} in cells alters the activity of Mg^{2+}-dependent enzymes, and thus causes lack of phosphorylation of protein-kinases and downregulation of synthesis of target proteins. The low level of K^+ channel transcription due to the shortage of Mg^{2+} in cells seems to indicate that the regulation of K^+ channels synthesis at the transcription level is performed through these Mg^{2+} levels.

In mammalian muscle cells the lack of K^+ and Ca^{2+} has two opposite effects: the shortage of Ca^{2+} causes spontaneous muscle contractions concomitant with ATP-hydrolysis, whereas low K^+ concentrations bring about weakening of myocardium contraction. Some spasmolytic mediators, such as dibazolum (2-benzil benzimidazolechloride – Fig. 7A), mimic artificially saturation by Ca^{2+}, temporarily stop muscle contractions, and slow down the pulsing of myocardium (Mashkovskyi, 1977). In yeast we observed a corresponding effect of an increased level of K^+ transcription in the absence of Ca^{2+}, and downregulation of the transcription by dibazolum or barbituric acid. cAMP-mediated stimulation of K^+ channel transcription was observed in rat cardiocytes but it brought about downregulation of the transcription in GH3 cells (Mori et al., 1993). In our experiments we tried to increase intracellular cAMP level by adding papaverinum (6,7-dimethoxy-1-(3,4-dimethoxybenzil)-isoquinoline – Fig. 7B) which is an alkaloid isolated from opium (Mashkovskyi, 1977). Similarly to dibazolum, this compound has spasmolytic effects on mammalian cells through inhibition of phosphodiesterase. By blocking the highly exergonic degradation of cAMP into AMP it affects the intracellular accumulation of cAMP, which in turn

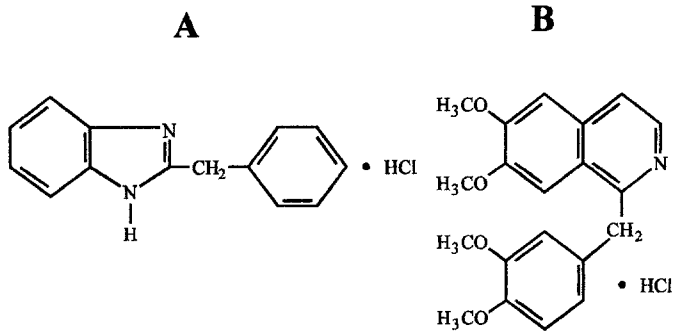

Fig. 7. A dibazolum (2-benzil benzimidazolechloride); **B** papaverinum (6,7-dimethoxy-1-(3,4-dimethoxybenzil)-isoquinoline)

regulates a variety of biochemical processes. The mechanism of the hypotensive effect of papaverinum on the human organism is not known but seems to go *via* initiation of the ATP-dependent efflux of Na^+ (concomitant diuretic and dehydration effect) and thinning of vascular wall swellings. A 4~5-fold higher level of the K^+ channel transcription in yeast in the presence of papaverinum was observed in both the full and the minimal media when K^+ concentrations changed from 7mM and 60μM. It is not clear whether this is due to the accumulation of cAMP acting as a positive regulator, or due to the ATP-dependent Na^+ efflux concomitant with the activation of K^+ channels.

The low number of transcribed K^+ channels in K^+ concentrations as low as 60μM, as well as the positive response to the higher pH, is in agreement with the expected traits of the 3d K^+ transporter in trk1Δtrk2Δ cells: the transcription is virtually pH-insensitive and under normal conditions does not participate in controlling the K^+ homeostasis of the cells (Ko and Gaber, 1991). Similar effects on the K^+ channel transcription regulation in yeast of the spasmolytic and hypotensive agents suggests that the K^+ channel functions are conserved.

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