Membrane Biology and Signal Transduction

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Reglatory volume decrease (RVD) of Opossum kidney (OK) cells depends on Ca^{2+}_i and a G-protein

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Hypotonic shock causes RVD of cultured OK-cells. During RVD the membrane-current relation was measured in the whole and slow whole cell patch clamp mode while simultaneously the cell diameter was recorded. At rest the membrane potential and membrane conductance depend not on cytoplasmic Ca^{2+} (Ca^{2+}_i) in the range of 10^{-8} M to 10^{-6} M, but RVD strongly depends on Ca^{2+}_i. For $Ca^{2+}_i < 10^{-7}$ M RVD is suppressed. Addition of 10 μM GTP[S] potentiates RVD. The time course of RVD becomes accelerated and the maximal change of cell diameter is reduced. Presence of GTP[S] shifts the Ca^{2+}_i - dependence of RVD to lower concentrations. During RVD the whole cell membrane current was repetitively recorded as continuous function of membrane potential for -60 mV to +60 mV. The high K-permeability observed at rest steeply decreases after a hypotonic shock. In addition cation unselective SA-channels become activated which cause rapid depolarization. During RVD the permeabilities for K^+, Na^+ and Cl^- increase and reach their maximum at maximal cell diameter while the total membrane surface area is left unchanged.

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VASOPRESSIN- AND CALCITONIN RESPONSIVENESS OF NA/H EXCHANGERS EXPRESSED IN CULTURED KIDNEY (LLC-PK1/PKE20) CELLS

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Cultured epithelial cells have been shown to express apical and/or basolateral Na/H exchange. Recently two distinct Na/H exchange activities have been demonstrated in LLC-PK1/PKE20 cells. The characteristics of these exchangers were found to vary both kinetically and in terms of regulatory control. On the basis of these observations we examined the effects of vasopressin (VP) and calcitonin (CT) on Na/H exchange activity of these cells. Na/H exchange activity was analyzed by microfluorometry in individual cells within a confluent monolayer, grown on a permeable support. Using BCECF, Na/H exchange activity was measured as the Na-dependent recovery of cytosolic pH from an acid load. Both maximal effective doses of VP and CT, when applied simultaneously from the basolateral and apical perfusion compartment, inhibited apical Na/H exchange and stimulated basolateral transport activity. Inhibition of apical Na/H exchange by VP was independent of the compartment containing the hormone, whereas stimulation of the basolateral Na/H exchange was only observed when the hormone was included in the apical perfusate. Interstingly, basolateral VP additions elicit an inhibitory response. Experiments on VP and CT action on cAMP formation demonstated that both hormones increased cAMP production irrespective of the side of application. It is concluded that LLC-PK1/PKE20 cells demonstrate a spatial separation of VP receptors: VP receptors coupled to the cAMP messenger system are located in the apical and basolateral membrane domain, whereas VP receptors coupled to the phospholipase C system are expressed in the apical membrane domain.

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VASOPRESSIN (VP)-INDUCED cAMP GENERATION IN ISOLATED TOAD BLADDER EPITHELIAL CELLS : INHIBITION BY AMILORIDE AND ETHYLISOPROPYLAMILORIDE (EIPA).

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It has been suggested that the observed inhibitory effect of amiloride on VP-induced increase in water permeability may be the result of the interference of this diuretic with the epithelial cAMP system. In this work, the effects of amiloride and of its analog EIPA on the VP-induced production of cAMP in isolated toad (*Bufo marinus*) bladder epithelial cells are presented. VP (50nM) induced an approximately 5-fold increase in cAMP production in both Na-containing and Na-free Ringer solutions. Both amiloride (1mM) and EIPA (10μM) inhibited the VP-induced stimulation of cAMP production, while having no effect on basal cAMP levels. However, the ability of EIPA to reverse the stimulation of cAMP production by VP was approximately 100-fold greater than that of amiloride. Dose-dependent cAMP production by forskolin (5μM) was unaffected by amiloride, a finding consistent with studies in toad bladder showing that this compound does not inhibit the forskolin-induced increase in water permeability. These results suggest that amiloride (and possibly EIPA) do not interfere with cAMP production at the level of the catalytic subunit of adenylate cyclase. Moreover, work is in progress to determine if this effect is due to a direct action on adenylate cyclase or to alterations via Na/H exchange inhibition.

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NET WATER FLUXES IN A6 CULTURED EPITHELIAL CELLS

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A6 cells cultured on permeable supports form high resistance epithelial sheets that respond to vasopressin (VP) by increasing net Na^+ transport; however, an increase in water transport has not been observed so far. We have now studied A6 cells cultured for 10 days on Millicell HA supports, and placed as a diaphragm in between glass chambers designed for automatic, net water flow (Jw) measurement. The basal side was exposed to isotonic amphibian Ringer and the apical side to the same Ringer diluted 10-fold : Jw was stable, averaging 0.08±0.01 $\mu l.min^{-1}.cm^{-2}$ (N=18), and was not increased by either VP or vasotocin. Since serosal hypertonicity (SH) induces a VP-like hydrosmotic effect, we also looked at its effect on A6 cells. When 200 mM mannitol was added to basal medium, Jw roughly doubled immediately. In addition, in 12 out of 18 experiments, Jw started to rise steadily after a lag time of 50±5 min reaching peak values of $2.5\mu l.min^{-1}.cm^{-2}$. Examination by freeze-fracture of cultured A6 cells exposed to SH revealed the presence of apical intramembrane particle aggregates, resembling those found in toad bladders, and alterations of tight junctions. These changes were not seen in control A6 cells. This is the first report of an increase in Jw accompanied by the appearance of apical aggregates in cultured epithelial cells. Further work will elucidate whether the SH-induced Jw correlates with the occurrence of the apical particle aggregates and/or with tight junction changes.

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INTRACELLULAR pH AND ACTIVE Na^+ TRANSPORT IN A6 MONOLAYERS: EVIDENCE FOR A RECIPROCAL RELATIONSHIP

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The pH_i was measured in A6 cells using the BCECF fluorescence method. The intracellular buffering power (β_{tot}) was determined in the presence of CO_2 while the regulatory processes (Na^+/H^+ and Cl^-/HCO_3^- exchangers) were suppressed. The measurements were performed at various pH_i and curves of β_{tot}, β_{CO2} and β_i ($\beta_i = \beta_{tot} - \beta_{CO2}$) versus pH_i were obtained. Under standard physiological conditions, pH_i was 7.22 ± 0.08 (n=8).

The electrical properties were also measured in these conditions; they were: short-circuit current, $I_o = 16 \pm 1$ μAcm^{-2}, $\Delta\Psi = 38 \pm 3$ mV and $R = 2.5 \pm 0.1$ $k\Omega cm^2$ (n=48).The I_o was measured as a fonction of the Na^+ concentration in the bathing fluid at 3 selected pH_i values (P_{CO2} 1.5, 6 and 20%). The data closely fitted to a hyperbolic shape, for each set. A plot according to Lineweaver-Burk revealed a mixed inhibition of I_o by decreasing pH_i. Amiloride, 2μM, a dose inhibiting the I_o by 80% and the Na^+/H^+ exchanger by 2%, lowered pH_i from 7.14 ± 0.06 to 7.09 ± 0.06 (n=28, $p<0.001$).The results suggest a role for pH_i in modulating the absorption of Na^+ across the apical and the basolateral membrane.

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Inward and outward rectifying K^+ conductances of the A6 cells basolateral membrane.

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In epithelial cells, the K^+ conductance (G_K) of the basolateral membrane is responsible for K^+ recirculation, and involved in the control of Na^+ transport and cell volume regulation. Contradictory results have been reported concerning the voltage dependence of this G_K. Two types of G_K have been described in basolateral membranes: a "basal" and a volume activated G_K. Both are blocked by barium and only the volume activated G_K is sensitive to quinidine. We have studied the voltage dependence of the A6 cells basolateral membrane G_K, under voltage clamp conditions, after the permeabilization of the apical membrane with amphotericin B. Under basal conditions (no Cl^- in the apical solution), the barium-sensitive G_K was inward rectifying (0.15±0.04 $mS{\cdot}cm^{-2}$ at -110 mV, and 0.05±0.01 mS/cm^2 at -30 mV) and insensitive to quinidine. When the cells were swollen (25 mM Cl^-) the G_K was much larger, quinidine sensitive and outward-rectifying (0.85±0.03 and 3.15±0.12 $mS{\cdot}cm^{-2}$ at -110 and -30 mV, respectively). In both cases the reversal potential of the barium-sensitive current was near the K^+ equilibrium potential (-79±3 and -77±1 mV). These inward and outward rectifying G_K could be generated by two types of K^+ channels with different voltage dependences.

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MINERALOCORTICOID SELECTIVITY IN TBM CELL LINE

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The sodium transport of the toad urinary bladder was reported to be selective for aldosterone. Selectivity was abolished by preincubation of carbenoxolone (Cbx 10^{-5}, 10 µM) a potent inhibitor of 11-ß-OHSD which prevents the conversion of corticosterone (cort) into its inactive metabolite (Gäggeler et al., Am.J.Physiol., 1989). In the present study we have examined whether TBM-18, clone 23 (TBM), an established cell line derived from the toad urinary bladder keeps the same mineralocorticoid selectivity in culture. The TBM cell line did not display any mineralocorticoid selectivity up to 8 hours after addition of corticosterone. When TBM cells were pretreated with cbx (10 µM), no difference in sodium transport response could be observed upon stimulation with cort (0.3 to 300 nM) and up to 10 hours of incubation. However, between 10 and 24 hours, a significant difference was observed as the cbx treated cells displayed twice as much sodium transport response (cort 3 or 30 nM) compared to the untreated one. Our data suggest that the level of 11-ß-OHSD is low in basal condition but can be induced in presence of cort, restoring partially mineralocorticoid selectivity.

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Na,K-ATPase EXPRESSION DURING CYTODIFFERENTIATION OF A TOAD BLADDER CELL LINE

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TBM 18-23 is a clonal cell line derived from the urinary bladder of the toad Bufo marinus. These cells undergo in culture a high degree of morphological and functional differentiation and express high resistance to ouabain (K_i=3.10^{-5}M) and responsiveness to aldosterone. Full length cDNAs for α_1 (3,4 kB) and $ß_1$ (2,3 kB) subunits of the Na,K-ATPase were cloned. The α_1 charged aminoacid distribution within the H1-H2 region involved in the ouabain-resistant phenotype is different from the rat α_1 isoform. A cDNA fragment coding for another ß isoform was also amplified using degenerated oligomers specific for $ß_2/ß_3$ isoforms. A near full length $ß_3$ cDNA (1.6 kB) was obtained and shares 80% homology with the Xenopus $ß_3$ subunit. By Northern blot analysis, it appears that $ß_3$ mRNA is expressed at a low level compared to that of $ß_1$. Preliminary data show that aldosterone do not upregulate $ß_3$ mRNA abundance by contrast to $ß_1$. During the cytodifferentiation process, the abundance of α_1, $ß_1$ and $ß_3$ mRNA and α_1 protein did not change during the first 8 days of culture despite large changes in Na transport and transepithelial resistance.

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An inducible expression vector for epithelial cells.

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ABSTRACT
A glucocorticoid-responsive vector is described which allows high inducible expression of cDNAs in stably transfected Madin-Darby canine kidney (MDCK) cell line. This vector (pLK-neo) is composed of a kidney MMTV LTR promoter, containing a glucocorticoid responsive element, a G418 resistance gene in a SV40 transcription unit and a polylinker insertion site for heterologous cDNAs. The polymeric immunoglobulin receptor (poly-Ig-receptor), the thymocyte marker (Thy-1), and a Thy-1-poly-Ig-receptor chimera were expressed in MDCK cells. High levels of mRNA accumulation in MDCK cells in response to dexamethasone was observed with a parallel 10 to 200 fold increase in protein synthesis. The dexamethasone response was dose dependent. This expression system permit us to study the trafficking of 3 different membrane proteins in a polarized epithelial cell system.

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A METHOD TO SELECT TRANSPORT MUTANTS IN RENAL CELL LINES: ^{32}P-SUICIDE AND REPLICA PLATING.

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We investigated whether ^{32}P-suicide and replica plating could be used to select Na-P_i transport mutants in renal cultured cells. Thus long storage of ^{32}P-heavily loaded LLC-PK_1 cells at -70°C showed that only cells that accumulated the label in the absence of Na survived after thawing, thus suggesting that Na-P_i transport-defective mutants could also survive. LLC-PK_1 cells also produced two replicas on polyester cloth. Since Na-dependent P_i uptake was expressed when cells grew on polyester, a great number of mutants can be quickly screened by assaying uptake on replicas followed by autoradiography. The method above can thus be used to select Na-P_i transport mutants in mutagenized LLC-PK_1 cells.

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EFFECTS OF MERCURY AND SILVER ON ELECTROPHYSIOLOGICAL PROPERTIES OF CULTERED RENAL EPITHELIAL CELLS

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Several metals are known to be nephrotoxic. Two epithelial cell lines (LLC-PK_1 and MDCK-I) with respectively proximal and distal nephron properties, grown at confluence on collagen coated filters were used to study the early mechanisms of functional alterations induced by metal ions. Short circuit current (I_{sc}) and transepithelial resistance (R_T) were measured using a modified Ussing chamber allowing continuous perfusion of medium (37°C) on the apical and basolateral sides. Inorganic mercury ($HgCl_2$) and silver ($AgNO_3$) applied to the apical side of the epithelia (10^{-7}-10^{-4} M), induced a marked increase of the basal I_{sc} : 2.5 and 5.5 fold for LLC-PK_1, 30 and 100 fold for MDCK-I cells, and a parallel decrease of R_T. For LLC-PK_1, this increase was dependent on the presence of small monovalent cations (Na^+, K^+, Li^+) at the apical side, but independent of the Na/glucose cotransporter. Thus, mercury and silver ions at very low concentrations induce an active transcellular ion transport probably by opening an electrogenic pathway for small cations in the apical membrane and decrease the R_T through an effect on the tight junctions. An interaction of these metal ions with SH-groups at the apical membrane appears a likely mechanism of these effects.

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DIFFERENCES IN ^{14}C-SALICYLIC ACID (SAL) MEMBRANE PERMEABILITIES IN INTACT MDCK-I CELL MONOLAYERS

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MDCK-I cells, grown on collagen coated filters, were used as a model of the distal nephron to study the nonionic diffusion of SAL. The transcellular SAL apparent permeability (P_{SAL}) and SAL intracellular content (SAL_i) were measured for both apical-to-basolateral (J_{SAL}^{AP-BL}) and the basolateral-to-apical (J_{SAL}^{BL-AP}) unidirectional fluxes. At 21°C, with 1 µM SAL, both fluxes were constant over a 10 min incubation period and were of same magnitude for identical pH conditions (pH 6.5->7.4). By contrast, SAL_i reached a plateau value after 6 min and was 15 times higher for J_{SAL}^{BL-AP} (0.105±0.008) than for J_{SAL}^{AP-BL} (1.642±0.057 pmol/µgDNA, n=5). Raising the temperature from 21° to 37°C resulted in an increase of P_{SAL} from 5.94±0.62 (n=4) to 31.56±4.29 $\cdot 10^{-7}$cm/s (n=6), but a decrease in SAL_i from 0.993±0.080 to 0.157±0.008 pmol/µgDNA/2 min when obtained from J_{SAL}^{BL-AP}. These data indicate that the SAL permeability of the AP membrane is lower and more sensitive to temperature than the BL membrane. We conclude that the AP membrane is the rate limiting step for SAL transcellular nonionic diffusion.

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EXPRESSION OF L-ALANINE TRANSPORT SYSTEMS OF RABBIT KIDNEY CORTEX IN XENOPUS LAEVIS OOCYTES.

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In the present study we report on the expression of Na-dependent (Na/Ala) and Na-independent (Ala) transport of L-Ala in X. laevis oocytes after injection of poly(A$^+$)RNA isolated from rabbit kidney cortex. Expression of Na/Ala and Ala transport was found to be time and dose dependent reaching maximal value (4-fold over the transport rate of water injected oocytes) within 5 days after injection of 40ng mRNA. Since total expressed Ala transport was not inhibited by aminoacetate (an inhibitor of amino acid metabolism), enhanced metabolization of Ala is ruled out. After fractionation on a sucrose gradient, highest expression of Na/Ala and Ala transport was observed with mRNA of 1.9 to 2.5 kb in length.Both expressed transport activities for Ala were almost completely inhibited by L-leucine. BCH (aminonorbornane carboxylic acid), a diagnostic reagent for the transport system of type L, inhibited Ala uptake by 50%. Furthermore, total Ala uptake was inhibited by approx. 80% by phenylalanine, but not by AIB.

It is concluded that injection of mRNA of rabbit kidney cortex leads to expression of at least two transport systems for L-alanine: of a system of type L and most likely of the neutral brush border (NBB) transport system.

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CLONING OF A PUTATIVE SUBUNIT OF THE EPITHELIAL SODIUM CHANNEL IN XENOPUS LAEVIS

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An amiloride-sensitive Na channel is localized to the apical membrane of high resistance epithelia. Antibodies directed against the fusion protein corresponding to a cDNA isolated from an A6 expression library recognize the sodium channel purified from A6 cells and from bovine kidney cells. The cDNA hybridizes to a 5 kb mRNA in A6 cells and in Xenopus oocytes. A 4.9 kb cDNA clone was isolated from a Xenopus ovary library and sequenced. It encodes a protein of 1420 amino acids. Hydropathy plot analysis reveals a single hydrophobic domain and 4 putative amphipathic helices including the hydrophobic domain. Injection of synthetic mRNA into Xenopus oocytes did not reconstitute an amiloride-sensitive sodium current. When A6 mRNA was coinjected, however, together with antisense oligonucleotides complementary to the cDNA sequence the amiloride-sensitive sodium current was completely abolished when compared to control oocytes injected with A6 mRNA with or without sense oligonucleotide. We conclude that this cDNA clone encodes one of the subunits of the epithelial sodium channel.

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ISOLATION OF THE GENE CODING FOR AN APICAL PROTEIN, WHICH IS A PUTATIVE SUBUNIT OF THE EPITHELIAL SODIUM CHANNEL IN XENOPUS LAEVIS

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A full size cDNA clone coding for an apical protein has recently been characterised by O.Staub et al. Expression studies in the Xenopus oocyte indicate that this protein might be a component of the amiloride sensitive sodium channel. We have isolated the corresponding gene from a Xenopus genomic library. It spans at least 30 kb. in length. We are currently establishing its structure by delineating the pattern of introns and exons. This knowledge will provide us with some hints on the functional domains of this putative component of the epithelial sodium channel. We are also sequencing the promotor area in order to identify cis-regulatory elements which might play a role in modulating the expression of the apical protein.

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PRESYNTHESIZED NA,K-ATPase SUBUNITS RETAIN THEIR ABILITY TO FORM STABLE, TRANSPORT COMPETENT α-β COMPLEXES.

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Assembly of α (Sα)- and β-subunits (Sβ) of NA,K-ATPase occurs at the level of the ER and is a prerequisite for the enzyme to become trypsin-resistant and to leave the ER. In this study, we have asked whether the formation of stable, transport competent α-β complexes depends on a coordinate synthesis of the two subunits or whether preexisting Sα (or Sβ) can associate to newly synthetized Sβ (or Sα). to study this question, we have used Xenopus oocytes which synthesize an excess trypsin sensitive Sα over Sβ. Injection of βcRNA , followed by pulse labeling with ^{35}S-methionine increases trypsin resistance of newly synthesized oocyte Sα. Similarly, presynthesized Sα, assessed after a 21h pulse and a 24h chase, can be stabilized by injection of βcRNA. Finally, Sβ, presynthesized from injected βcRNA becomes fully glycosylated after injection of αcRNA, in contrast to Sβ synthesized alone which remains coreglycosylated. Our data indicate that a synchronized synthesis of Sα and Sβ is not a prerequisite for a correct oligomerization of Na,K-ATPase but that newly synthesized subunits retain a configuration competent for assembly over a prolonged time.

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SITE DIRECTED MUTAGENESIS OF β-SUBUNIT: EFFECTS ON STABILIZATION AND ER EXIT OF NA,K-ATPase

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In order to better understand the structure-function relationship of the β-subunit (Sβ) of Na,K-ATPase, we have expressed Sβ-mutants in Xenopus oocytes and have studied their ability to stabilize the catalytic α_1-subunit ($S\alpha_1$) by forming α-β complexes which can leave the ER and become fully glycosylated. Site directed mutagenesis was carried out on a highly conserved sequence among β-isoform ectodomains: Y242 - Y or F - P - Y - Y246. In contrast to $S\beta_1$ wild type, the P244G mutant cannot render $S\alpha_1$ trypsin-resistant or become fully glycosylated. On the other hand, Y243F and Y246F mutants were indistinguishable from wild type $S\beta_1$. Mutations in the 1st (N158D) and 2nd (N193D) glycosylation site of $S\beta_1$ permit coreglycosylation but the N193D mutant does not allow for further glycosylation processing or stabilization of the α-subunit. These results support the hypothesis that the formation of stable, transport-competent Na,K-ATPase strongly depends on the proper association of Sα and Sβ.

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VOLTAGE DEPENDENCE OF THE ACTIVATION OF THE NA-K-PUMP BY POTASSIUM. Horisberger, J.-D. Institut de Pharmacologie et de Toxicologie de l'Université, Lausanne.

The activity of the Na-K-pump depends on the membrane potential, but it is not clear which steps of the pump cycle are voltage dependent. We have studied the effect of membrane potential on the activation of the pump by K^+. Stage V-VI oocytes of Xenopus Laevis were first loaded with Na^+ by 15 min exposure to K-free solution containing 100 µM nystatin. After removal of the nystatin, whole cell currents were measured by the two-electrodes voltage clamp method, in the presence of 5 mM Ba^{++} to block all K^+ channels. The currents induced by K^+ concentrations from 0.3 to 10 mM were measured at holding potentials between 0 and -120 mV. By fitting the data to the Hill equation, we observed that the apparent K_m for K^+ increased with membrane hyperpolarization (from 0.82 ± 0.03 mM at 0 mV to 1.86 ± 0.21 mM at -120 mV), while the Hill coefficient was constant around 1.7. This voltage dependence is opposite to that expected for a K^+ binding site located in the membrane electrical field. The higher apparent affinity with depolarization might result from a voltage-induced shift from the E1 to the E2 conformation, the form which has a high affinity for K^+. Membrane potential must be controlled when precise measurements of K^+ affinity are needed.

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INTRACELLULAR INHIBITION OF Na/K-ANTIPORT BY $HgCl_2$ DEMONSTRATED IN TWO-SIDED LIPOSOMES

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Liposomes filled with Na-Mg-ATP containing pure Na,K-ATPase molecules in random orientation (50% inside-out, 50% right-side out) have been used successfully for determining the potency, membrane permeability and side of action of exo- and endogenous Na,K-ATPase inhibitors. Although Hg(II)-compounds are powerful natriuretic drugs their molecular action mechanism is still unknown. By comparing the kinetics of the inhibition of the right-side-out with the inside-out oriented Na,K-ATPase molecules by externally added $HgCl_2$, the side of action can be determined. Inhibition takes place first at the intracellular surface in contrast to the extracellular action mechanism of the cardioactive steroids. Thus, in the cell, Hg(II) must penetrate the membrane to block the Na/K antiport. This finding predicts that, in vivo, only membrane permeable Hg(II)-compounds are potential Na,K-ATPase inhibitors and thereby natriuretic drugs.

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REVERSIBLE STRUCTURAL MODIFICATION OF PURIFIED Na,K-ATPase BY $HgCl_2$

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Hg(II) but not Cd, Cu, Pb and Zn blocks the ATPase activity and Na/K-antiport catalyzed by purified Na,K-ATPase in the presence of 1 mM EDTA with an IC_{50} of 200 nM. The enzyme inhibition is prevented by 1 mM DMPS and 70-80% activity is recovered if DMPS is added after Hg(II). No crosslinks betweeen alpha and beta subunits of the Na,K-ATPase molecule are detected by PAGE. Tryptic mapping of native and Hg(II)-modified enzyme shows exposure of new trypsin-sensitive bonds of the alpha-subunit. Trypsinolysis in the presence of Na ions ($E1$-form) does not diminish the beta subunit area; in the presence of K ions (E_2-form), however, the area of the beta subunit is decreased too, indicating alteration of membrane anchoring. In conclusion, Hg(II) profoundly and reversibly modifies the Na,K-ATPase structure in vitro; our results suggest that some body Na,K-ATPase could undergo such structural alterations in vivo as the normal Hg(II) concentration is around 20 nM.

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EVIDENCES FOR THE PRESENCE OF A LUMINAL URATE/ANION EXCHANGER IN THE PROXIMAL TUBULE OF THE HUMAN KIDNEY

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Urate undergoes bidirectional transport in proximal tubules of mammalian kidneys. Depending on the species, either net reabsorption or net secretion is observed. Previous studies using brush border membrane vesicles (BBMV) demonstrated the presence of a luminal urate/anion exchanger in reabsorbing species (rats, dogs), which is absent in secreting species (rabbits, pigs). We investigated if a urate-anion exchanger was also present in BBMV of man, a urate reabsorber. Renal material was obtained from one patient undergoing nephrectomy for renal malignancy and tumor-free cortex was used for the isolation of BBMV. The specific activity of the luminal marker leucine-aminopeptidase was enriched 8.7-fold in BBMV. Compared to pH equilibrium (pHin=pHout=6.0), an in-to-out OH^- gradient (pHin=7.4; pHout=6.0) stimulated the 15s uptake of 38 µM ^{14}C-urate more than 2-fold and an overshoot of 113 % was observed at 2 min. Under both voltage and pH clamps, a Cl^- gradient (in=40 mM; out=6.7 mM) enhanced the 15s uptake of 49 µM ^{14}C-urate by 1.4-fold compared to Cl^- equilibrium (in=out=40 mM). These data indicate the presence in BBMV from human kidney cortex of an anion exchanger having affinity for urate, OH^- and Cl^-. This transport system might be responsible for urate reabsorption in human proximal nephron.

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BASOLATERAL Na+/DICARBOXYLATE COTRANSPORT AND DICARBOXYLATE/BILE ACID EXCHANGE IN RAT HEPATOCYTES.

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To further characterize the mechanisms and driving forces involved in hepatocellular organic anion uptake we investigated the relationship between transport of the endogenous dicarboxylate α-ketoglutarate (αKG) and the unconjugated bile acid cholate (CA). In primary cultured hepatocytes 75% of total CA uptake occured via a Na+ independent, DIDS sensitive pathway, which was saturable (K_m=7.4µM) and cis-inhibited by αKG (1mM). However, in the presence of an inwardly directed Na+ gradient extracellular αKG (10µM) stimulated rather than inhibited CA uptake suggesting coupled Na+/αKG cotransport and αKG/CA exchange. This hypothesis was further tested in isolated basolateral liver plasma membrane vesicles. The studies demonstrated a) the presence of Na+ dependent αKG uptake ; b) Na+ independent trans-stimulation of CA uptake by intravesicular αKG and p-aminohippurate (PAH), and c) stimulation of total CA uptake in the presence of Na+ by the addition of αKG (10µM) to the extravesicular medium. These studies support the concept of concomitant Na+/αKG cotransport and αKG/CA exchange. Since the latter also accepts PAH, dicarboxylate dependent anion exchange might mediate hepatocellular uptake of cholephilic organic anions other than cholate as well.

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MODIFICATION OF THE STIMULATORY ACTION OF VASOPRESSIN ON THE HEPATIC Na^+ PUMP

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Previous own results have shown that vasopressin (AVP) stimulates uptake of $^{22}Na^+$ into plasma membrane vesicles isolated from rat liver. Others have suggested that elevated (Na^+, K^+)ATPase-mediated transport activity observed in rat hepatocytes exposed to AVP may involve protein kinase C (PKC). The mechanism of the hormonal activation of the Na^+ pump was further studied with phosphatidylinositol-specific phospholipase C (PLC) and agents affecting PKC activity. Vanadate-sensitive uptake of $^{22}Na^+$ by rat liver plasma membrane vesicles was measured. PLC (0.03 U/ml) enhanced the stimulatory effect of AVP (100 nM) but had no effect in the absence of the hormone. By the addition of 10 nM staurosporine (inhibitor of PKC) the effects of AVP and PLC were further enhanced whereas 5 µM phorbol myristate acetate suppressed the stimulation by AVP. Our results indicate that phosphoinositide breakdown enhances and activation of PKC impairs the effect of AVP on the hepatic Na^+ pump.

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G0-G1 CELL CYCLE PHASE SHIFT IS DIFFERENTIALLY INDUCED BY LIVER MITOGENS IN CULTURED HEPATOCYTES

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One of the initial responses of cells when exposed to a mitogenic chemical is the shift from the G0 to the G1 cell cycle phase. The two phases can be distinguished in nuclei of resting cell populations by their quinacrinedihydrochloride fluorescence quantified by image analysis. The method was further validated in rat hepatocyte cultures. EGF increased the number of decondensed (G1) nuclei whereas retinoic acid increased the number of condensed ones (G0). Exposure (24-48h) to three liver mitogens, phenobarbital (PB), thioacetamide (TA) and cyproterone acetate (CPA) showed that CPA induced the highest decrease in nuclei fluorescence in the 2N (up to 90%) and TA in the 8N nuclei. PB affected all three ploidy classes similarly. The induced decondensation patterns were further modified by the exposure level and exposure interval (24-72h). The cell cycle shift seems to be an early and rapidly measurable indicator for the detection of agents which interfere with growth processes.

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DIFFERENT SENSITIVITY OF CARBONIC ANHYDRASE (CA) TO DEUTERIUMDIOXID (D_2O) IN HEMOLYSATE (H) AND RAT LIVER CYTOSOL (RLC)

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Bicarbonate secretion is essential for formation of bile salt-independent bile and the hypercholeresis induced by ursodesoxycholic acid (UDCA); heavy water reduces the UDCA-induced bicarbonate secretion in the isolated perfused rat liver (J Hepat 11:S30; 1990). Therefore, we analysed the effect of D_2O on carbonic anhydrase, a source of bicarbonate, in rat liver cytosol and, for comparison, in hemolysate. The effect of UDCA on RLC CA activity was also investigated. In 100 % D_2O medium CA activity in hemolysate was reduced by 75% compared to a H_2O medium (14.1 ± 2.1 vs. 3.9 ± 2.0 WAU/mg Hb, $p<0.01$). In contrast, RLC CA activity was only reduced by 35% in a 100% D_2O medium (1.4 ± 0.3 vs. 0.9 ± 0.3 WAU/mg protein, $p<0.05$). While the D_2O effect in H was dose-dependent ($ED_{50} \approx 65\%$) RLC CA was affected at 100% D_2O, only. 100 µM UDCA in the medium reduced RLC CA activity by 30%, (1.4 ± 0.4 vs. 0.9 ± 0.4 WAU/mg protein, $p<0.05$). Thus, erythrocyte and liver cytosol CA differ in their sensitivity against D_2O. Since cytosolic liver CA is inhibited by UDCA, but not by D_2O, at concentrations inhibitory in vivo, it appears unlikeley that CA is involved in UDCA-stimulated HCO_3-secretion.

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CYCLOPIAZONIC ACID INHIBITS THE Ca^{2+}-ATPase OF SARCOPLASMIC RETICULUM IN A7r5 CELLS

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The mycotoxin cyclopiazonic acid (CPA) is produced by certain *Aspergillus* and *Penicillium* genera and may be found as natural contaminant of corn, peanuts and certain mold-fermented cheeses. The compound has been identified as an inhibitor of the SR-Ca^{2+}-ATPase in skeletal muscle (Goeger et al.,Biochem.Pharmacol. 37, 978-981, 1988). We tested the compound on Ca^{2+}-uptake and agonist-induced Ca^{2+}-efflux in smooth muscle cells of the A7r5 cell line. CPA inhibited the filling of agonist sensitive pools with a $pIC_{50}=6.03$. A stimulatory effect on passive Ca^{2+}-efflux indicating a continuous turnover of calcium between the stores and the cytosol was also noticed. CPA, above 10 µM, favors Ca^{2+}-efflux from the cell by the sarcolemmal Ca^{2+}-ATPase. Agonist stimulated efflux is enhanced by CPA, reflecting a larger efflux from the cytosol, since reuptake into pools is inhibited. CPA appears to be a valuable tool for elucidating cellular mechanisms of Ca^{2+}-handling.

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DIFFERENTIATION INDUCING FACTOR FROM THE SLIME MOULD INHIBITS Ca2+ ENTRY INTO MAMMALIAN SMOOTH MUSCLE CELLS

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Differentiation inducing factor (DIF-1) induces amoebae cells from the slime mould *Dictyostelium discoideum* to differentiate into stalk cells. The structure of DIF-1 has been elucidated (Morris et al., Nature 328, 811-814, 1987). As changes in cytosolic Ca^{2+} are involved in morphogenesis, DIF-1 tested with respect to Ca^{2+} handling in the A7r5 rat smooth muscle cell line. Vasoconstrictor (*vasopressin, endothelin, or angiotensin II*)-stimulated $^{45}Ca^{2+}$-influx into the cells was inhibited by DIF-1 ($IC_{50} \approx 1$ µM). The late sustained rise in intracellular [Ca^{2+}] upon agonist stimulation (as measured with fura-2) was also inhibited as was the contraction of resistance vessels. These activities of DIF-1 resemble those of a blocker of receptor-operated calcium channels. Although DIF-1 induces differentiation of the mould at lower concentrations (nM range), it appears to be a valuable tool for the study of Ca^{2+} handling in these mammalian cells.

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ELECTRICAL PROPERTIES OF GAP JUNCTIONS STUDIED IN PAIRS OF INSECT CELL

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Cells of an insect line (Aedes albupictus) were grown in culture at low density (~ 10^5 cells/cm^2). Pairs of cells spontaneously formed were selected to study the intercellular conductance (g_j). The experimental approach adopted involved a double voltage-clamp method in conjunction with patch pipettes. Two kinds of cell-to-cell contacts were observed, gap junctions and cytoplasmic bridges, existing individually or in combination. In case of gap junctions, g_j exhibited time- and voltage-dependent modulations. Instantaneous values of g_j were not dependent on the trans-junctional voltage gradient (V_j) and the non-junctional membrane potential (V_m). Steady-state values of g_j decreased at $V_j > \pm 20$ mV and at V_m more positive than -40 mV. In addition, g_j was reduced reversibly by exposure to 3 mM heptanol. Such cell pairs, when weakly coupled, revealed a gap junction channel conductance of 60-200 pS. In case of cytoplasmic bridges, g_j remained constant, i.e. it was not affected by V_j, V_m, or heptanol. Supported by SNSF (31-25333.88).

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MODULATION OF CARDIAC GAP JUNCTIONS BY PHORBOL ESTER

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Myocytes were isolated from neonatal rat hearts and grown in tissue culture. Spontaneously formed cell pairs were used to assess the conductance of gap junctions (g_j) and their channels (γ_j). The measurements were performed with a double voltage-clamp method involving patch pipettes (Ca-aspartate,110; NaCl,10; $MgCl_2$,1; TEA-Cl,10; HEPES,5; EGTA,10; $CaCl_2$,1; pCa ≈ 8; Mg-ATP,3; Na-cAMP,0.1;) and whole-cell, tight-seal recording. Exposure to 125 nM of the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate) impaired the current flow (I_j) across gap junctions within minutes. Examination of the microscopic currents suggested that the decrease in g_j is caused by a modification of the channel kinetics rather than by γ_j. Analysis revealed a mean γ_j of 56 pS. These effects of TPA on g_j were not seen when Ca^{2+} in the pipette solution was elevated (EGTA,5; $CaCl_2$,2.5; pCa = 6.8). (supported by SNSF #31-25333.88)

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FLASH-PHOTOLYSIS OF 'CAGED' CALCIUM ACTIVATES Na^+Ca^{2+}-EXCHANGE CURRENTS IN CARDIAC MYOCYTES

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Rapid concentration jumps of intracellular Ca^{2+} ($\tau \approx 200$ µs) were performed with UV-flash photolysis of caged Ca^{2+} (DM-nitrophen) in single Guinea pig cardiac myocytes while membrane current was recorded using the whole-cell patch-clamp technique. Photorelease of Ca^{2+} induced a rapidly activating ($\tau \approx 400$ µs) inward current and a twitch contraction. The inward current decayed monoexponentially ($\tau \approx 400$ ms) while the Na^+Ca^{2+} exchanger extruded Ca^{2+} from the cytosol, as indicated by relengthening of the cell. The Na^+Ca^{2+} exchanger is known to generate net inward current when removing Ca^{2+} from the cytosol because it exchanges 1 Ca^{2+} for 3 Na^+ ions. We identified the Na^+Ca^{2+} exchanger as the source of the observed inward currents based on the voltage-dependence and the sensitivity to organic and inorganic inhibitors (e. g. DCB, Ni^{2+}, La^{3+}, zero $[Na^+]_o$). Under experimental conditions designed to slow down the exchanger, we were able to resolve transient current phenomena on a sub-millisecond time-scale. Conformational rearrangements of the exchanger molecules (following Ca^{2+} binding) are probably responsible for these currents. Thus, with this approach the molecular dynamics of the Na^+Ca^{2+} exchanger can now be studied for the first time. (Supported by SNF and NIH).

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ENGINEERING OF A MOLECULAR DEVICE FOR LIGHT-DRIVEN CHARGE DISLOCATION

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The two terminal transmembrane segments (V2) of bacteriorhodopsin (bR) provide physicochemical and structural features for the construction of a light-dependent molecular device. The inherently stable peptide building block derived from the thermostable archaebacterial protein includes the chromophore binding site (Lys 216) and essential parts of the chromophore binding pocket (Trp 182, Trp 189). Following protease V8 fragmentation of permethylated purple membranes the V2 peptide has been isolated and selectively acetylated at its N-terminal end. Chromophores (all-trans retinylisothiocyanate, azobenzene-isothiocyanates) which undergo light-dependent cis/trans isomerization were covalently linked to Lys 216 by thioarbamoylation. Conversion of incident light to molecular motion, visualized by molecular modeling, has been investigated by absorption spectroscopy in membrane mimetic systems.

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LAGRANGIAN THERMODYNAMICS OF (BIO)CHEMICAL NETWORKS

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A new approach to the thermodynamics of chemical networks based on variational principles of the Hamiltonian type and valid far from thermodynamic equilibrium, the appropriate biological condition, is presented. Previous approaches have been restricted to either the near equilibrium regime or the neighborhood of a stable stationary state far from equilibrium or have not encompassed chemical reactions. An energy function containing mass balance constraints is constructed with the help of Nöether's theorem, and it is shown that, for the appropriate Lagrangian, the set of equations describing chemical kinetics correspond to a maximum of this (nonequilibrium) energy function with respect to the vector of reaction rates. For other sets of independent variables the maximum of this energy function becomes an extremum of the appropriate extended thermodynamic potential. For steady states close to thermodynamic equilibrium this approach reduces to Onsager's theorem of minimum dissipation.

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ESTABLISHMENT OF A T-LYMPHOBLASTOID CELL LINE DEFICIENT IN GALACTOSYLTRANSFERASE ACTIVITY

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Red blood cells from a patient (R.R.) with permanent mixed-field polyagglutinability expose the Tn antigen (ÁGalNAc) due to galactosyltransferase (gal-T) deficiency instead of the normally occurring TF antigen (Galß1-3GalNAc). To delineate the extent of this enzyme deficiency in hemopoiesis, peripheral blood lymphocytes (PBL) from patient R.R. were investigated for the occurrence of Tn or TF antigens with monoclonal antibodies (MAB's) and flow cytometry. In addition, MAB's to B (CD19), T (CD3) and natural killer (NK) cells (CD16) were used in double staining experiments in order to test different subsets for gal-T deficiency.
In contrast to normal donors, PBL from R.R. contain a TF antigen-negative subpopulation (20%-25%) which consists of both non-B/non-T cells (NK?) and T cells. A sizable fraction of these two subsets also stains for Tn antigen. B cells from R.R. seem to be less affected from PMFP.
TF antigen-negative lymphocytes were isolated by FACS. 24 T cell clones were established by limiting dilution and stimulation with PHA and IL-2. 14 clones expressed Tn antigen as shown by immunofluorescence. These Tn-lymphoblasts were deficient in gal-T activity. Conversely, the other clones expressing TF antigen contained gal-T activity. These cell lines will be instrumental in understanding this enzyme deficiency. This study was supported by grant 3.136.088 of the SNSF.

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EXPRESSION OF THE SURFACE METALLOPROTEASE OF *LEISHMANIA MAJOR* DURING DIFFERENTIATION *IN VITRO*.

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During its life cycle, the protozoan parasite *Leishmania* exists either as a flagellated promastigote in the insect vector or as an intracellular amastigote in the mammalian host. Amastigotes isolated from lesions were allowed to transform into promastigotes *in vitro*. The expression of the surface metalloprotease (PSP) was followed throughout differentiation using immunoblots, biosynthetic labeling with ^{35}S-methionine, surface iodination and zymograms on fibrinogen-containing gels. Amastigotes contain less than 1% of the protease activity detected in the promastigote. The expression of the protease begins 4 hours after the onset of differentiation and is completed after 12 hours. This synthesis preceeds the complete morphological differentiation to the promasigote stage.

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In vitro and in vivo studies of adhesion of Phytophthora infestans to polystyrene and to its host

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Physical contact between fungal pathogens and their plant hosts may lead to interactions of surface molecules resulting in adhesion, recognition and induction of pathogenesis-related events.
We used a model system to study the inhibition of fungal germlings treated with various detergents, enzymes and lectins on a polystyrene surface. The results of these inhibition studies suggest that a glycoprotein could be important for adhesion.
These results were confirmed on the host with infection of potato leaves with pretreated cysts. To characterize the glycoprotein, we have examined the extracellular proteins from liquid cultures of the fungus by SDS-PAGE and probing with peroxidase-labelled lectins.

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IAA EFFLUX CARRIER STUDIED BY IMPEDANCE SPECTROSCOPY

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Recently, a method has been developed for determining simultaneously open channel density and conformational changes in membrane channel proteins. It relies on measuring the low-frequency impedance spectrum of the membrane deposited using the Langmuir-Blodgett technique onto a silicon wafer to form an electrolyte-membrane-semiconductor structure (EMS). Changes in conformation and hence net dipole moment of the membrane protein alter the capacitance of the semiconductor depletion layer, whereas the opening of channels alters the membrane conductivity. Both these parameters may be determined from the impedance spectrum using an appropriate model. In the present realization, membrane fractions from maize root segments were investigated. Marked changes in the impedance spectrum of the EMS were observed when indole acetic acid (IAA), or inhibitors of IAA transport, or both were added to the electrolyte. The results are discussed in terms of the activity of the IAA efflux carrier.

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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF THE 14 AND 26 kDa PHOSPHORYLATED POLYPEPTIDES LOCATED IN THE ENVELOPE OF SPINACH CHLOROPLAST

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Phosphorylation activities are found in the envelope of spinach chloroplasts. The major phosphorylated polypeptides have apparent Mr of 14, 26 and 67 kDa. The 67 kDa spinach phosphoprotein (equivalent to the pea 64 kDa) has been recently identified to be a phosphoglucomutase (Salvucci et al. (1990) Plant Physiol. 93, 105-109), whereas the 14 and 26 kDa phosphoproteins have not yet been precisely identified. Athough their electrophoretic mobilities suggest that the 14 kDa polypeptide could be a pool of phosphorylated mature Rubisco small subunit (Soll J. and Buchanan B. (1983) J.B.C. 258 (11), 6686-6689) and the envelope 26 kDa phosphoprotein one form of the light-harvesting chloroplast protein complex, no strong biochemical evidences are available so far to assess their precise nature. Thus, the purpose of this study was to characterize these two phosphoproteins, using immunological and 2-D electrophoresis techniques. Furthermore, in order to understand the physiological role of the 14 and 26 kDa polypeptides in the chloroplast, we have also analysed envelope phosphorylation by modulating some biochemical parameters such as pH and presence or absence of Ca2+ and Mg2+. Our results indicate that in vitro labelling of both polypeptides in presence of(32P)ATP was regulated by Ca2+ and displayed the same pH dependency.

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ATP CONTENT OF GERMINATING SEEDS IN RELATION TO SEED QUALITY.

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Since a long time, agronomists have attempted to find parameters which at an early stage could unambiguously predict the quality of seed lots. The most used parameter is the percentage of germination (GP). However, its determination is time consuming. Thus, biochemical parameters such as the content of ATP have been proposed to express seed quality. The value of this biochemical "marker" is unfortunately controversial. In this investigation, we study the relationship between GP and the ATP content together with seed respiration. Results show that (1)The imbibition of seeds was necessary to detect ATP content and 02 uptake; (2)As a function of imbibition time, both ATP content and seed weight increased progressively up to a plateau, the beginning of which varied with seed species; (3) Respiration activity of seed displayed a similar pattern. (4)There is a good correlation (r>0.9) between GP and ATP content. This was verified under the following conditions: by mixing seed lots displaying low and high GP, by analyzing 7 random onion samples and by comparing lettuce seeds stored at different temperatures. These results open interesting perspectives on the possibility of monitoring the quality of seeds during their storage by ATP content.

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The sorting receptor for soluble ER proteins

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One of the sorting problems a eucaryotic cell faces in the Endoplasmic Reticulum (ER) is how to separate the secreted proteins from the soluble, resident ER proteins. Previously it has been shown that luminal ER proteins (e.g. BiP, GRP94, PDI etc.) share a common C-terminal tetrapeptide (KDEL in animal cells, HDEL in *S. cerevisiae*) which is necessary and sufficient for their retention in the ER.
The sorting receptor from yeast has been cloned by a genetic approach. It determines the capacity as well as the specificity of the retention system. It is a 26 kD membrane protein with seven trans-membrane segments and is essential for growth in yeast.
Using two receptors with different specificities from two yeast species, chimeric receptors were constructed and the specificity-determining region was localized close to the N-terminus. This region must be in the lumen of the ER in order to interact with the retention signal. Based on this assumption and on hydropathy plots, the membrane orientation of the receptor has been predicted.
Supported by Roche Research Foundation.

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BIOSYNTHESIS OF MANNOSYLINOSITOLPHOSPHOCERAMIDES IN SACCHAROMYCES CEREVISIAE IS DEPENDENT ON GENES CONTROLLING THE FLOW OF SECRETORY VESICLES FROM THE ENDOPLASMIC RETICULUM TO THE GOLGI

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Saccharomyces cerevisiae contains several abundant phosphoinositol containing sphingolipids, namely inositolphospho-ceramides (IPC's), mannosylinositol-phosphoceramide (MIPC) which is substituted on the headgroup with an additional mannose, and $M(IP)_2C$, a ceramide substituted with one mannose and two phosphoinositol groups. Using well defined temperature-sensitive secretion mutants, we demonstrate that the biosynthesis of MIPC, $M(IP)_2C$ and a subclass of IPC's is dependent on genes which are required for the vesicular transport of proteins from the ER to the Golgi. Synthesis of these lipids in intact cells is dependent on metabolic energy. A likely but tentative interpretation of the data is that the biosynthesis of these sphingolipids is restricted to the Golgi, and that one or more substrates for the biosynthesis of these sphingolipids are delivered to the Golgi by an obligatory vesicular step. Alternative models to explain the data are also discussed.

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A NOVEL APPROACH TO EXPRESS HETEROLOGOUS MEMBRANE PROTEINS IN S.CEREVISIAE

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The enzyme superfamily comprising cytochrome P450 enzymes is responsible for the biotransformation of promutagens to mutagens. In order to use S. cerevisiae as an in vitro genotoxicity test system capable of intracellular promutagen activation we intend to express particular important human P450 genes in that organism. Expression of these microsomal enzymes might be limited by the low amount of endoplasmic reticulum present in yeast. Overexpression of *HMG1* in yeast has been shown to result in ER-like membrane proliferations. By expressing a hybrid gene consisting of *HMG1* sequences, human P450-reductase sequences and *P450IA1* sequences we expect three consequences: (1) additional internal membranes will be synthesized, (2) the chimeric protein will integrate in these membranes and (3) exhibit cytochrome P450 specific activity due to homologous interaction with the P450-reductase moiety. Preliminary data will be shown.

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CHARACTERIZATION OF HUMAN INTERLEUKIN 3 AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RECEPTORS ON THE HUMAN LEUKEMIA CELL LINE M-07: EVIDENCE FOR DISTINCT AND SHARED MEMBRANE RECEPTORS

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The biologic response of the human leukemia cell line M-07 to granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) is mediated by a low number of high affinity receptors (kD ~ 100 pM). Cross-competition studies revealed that IL-3 and GM-CSF partially inhibited specific binding of the heterologous radiolabeled ligand. The molecular mechanism of these findings was investigated by chemical crosslinking and immunoprecipitation. Crosslinking with radiolabeled ligands gave a trimolecular membrane complex consisting of a major 73 kDa and two minor 120 and 128 kDa membrane proteins for IL-3, and a major 84 kDa and two minor 120 and 130 kDa proteins for GM-CSF. Based on saturation and competition studies it is concluded that the major 73 kDa and 84 kDa protein are involved in low affinity binding for IL-3 and GM-CSF respectively, whereas the minor, higher molecular weight proteins have high affinity binding.

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NEUTROPHIL-ACTIVATING PEPTIDE RECEPTORS

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Neutrophil-activating peptide 1 and 2 (NAP-1/IL-8 and NAP-2) and gro/melanoma growth-stimulatory activity (gro/MGSA) are structurally related inflammatory cytokines with similar neutrophil-activating properties. In order to investigate the interaction of these peptides with receptors on human neutrophils NAP-1/IL-8 was enzymatically iodinated. Each [125]I-NAP-1/IL-8 sample was characterized by self-displacement analysis to evaluate its binding capacity (30-40%) and to determine its specific radioactivity (0.3-1.3x10E+18 dpm/mol unmodified NAP-1/Il-8 equivalents). Binding of [125]I-NAP-1/IL-8 (0.2 nM) to neutrophils (2x10E+6) was rapid: 80% of maximal binding was obtained after 5 min at 0-4°C, and equilibrium was reached after 45-60 min. After incubation at 0-4°C for 3 h, over 95% of bound peptides could be recovered by acid treatment. Scatchard analysis revealed 64,500±14,000 NAP-1/IL-8 receptors per cell with a KD of 0.18±0.07 nM. Competition with increasing concentrations of unlabeled NAP-2 and gro/MGSA resolved two classes of NAP-1/IL-8 binding sites: About 70% of them bound NAP-2 and gro/MGSA with high affinity (KD = 0.34±0.20 and 0.14±0.02 nM), while 30% were of low affinity (KD = 100±20 and 130±10 nM). Binding sites of different affinities were not apparent upon competition of [125]I-NAP-1/IL-8 with unlabeled NAP-1/IL-8.

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TWO NOVEL PROTEIN TYROSINE KINASES EACH WITH A SECOND PHOSPHOTRANSFERASE-RELATED CATALITIC DOMAIN DEFINE A NEW CLASS OF PROTEIN KINASE.

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We have exploited the existence of two highly conserved sequence elements within the catalitic domain of protein tyrosine kinases(PTKs) to generate PTK-specific degenerate oligonucleotide primers. By application of the polymerase chain reaction, portions of the catalitic domains of several novel PTKs were amplified. We describe the primary sequence of two of these new PTKs,JAK1 and JAK2, members of a new class of PTK characterized by the presince of a second phosphotransferase-related domain immediately N-terminal to the PTK domain. The second phosphotransferase domain bears all of the hallmarks of a protein kinase, although its structure differs significantly from the PTK and serine/threonine kinases. JAK1 encodes a large, membrane-associated phosphoprotein of approximately 130,000 daltons.

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NOVEL PROTEIN KINASE GENES IN MAMMARY CELLS IDENTIFIED BY PCR-CLONING

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We are interested in the role of protein tyrosine kinases (PTKs), in mouse-mammary-gland differentiation and development. As a model we are using a mammary-gland-derived cell culture system consisting of fibroblastic (30F) and epithelial (31E) cell clones. Upon co-culture, the epithelial cells are able to differentiate both morphologically and functionally. We have synthesized cDNA from 30F, 31E and E/F mixed cultured cells and performed PCR with primers corresponding to highly conserved regions within the catalytic domain of protein kinase (PK) genes. We have identified more than 16 different PK genes expressed in these cells, at least 5 of which, including two PTK specific clones, show no significant homology to known genes. All of the clones characterized so far show a discrete organ specific expression. Using a second set of nested primers for reamplification, we were able to select for receptor-like tyrosine kinases. In an attempt to get epithelial cell specific clones, we are devising a method for direct subtractive cloning of PCR-material, involving biotin labelling during PCR-amplification, subtractive liquid hybridisation and biotin/avidin affinity chromatography.

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INFLUENZA HAEMAGGLUTININ-INDUCED MEMBRANE FUSION: THE DELAY AFTER pH REDUCTION DEPENDS ON THE HAEMAGGLUTININ SURFACE DENSITY

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We have studied the early stages of influenza virus fusion with erythrocyte membranes, as well as fusion of erythrocytes with cell membranes expressing the influenza haemagglutinin (HA). In both cases studied there is a pH dependent time lag before the onset of fusion, but of an order of magnitude difference (<4 sec vs >30 sec). Two approaches were used to show that the difference in time lag depends on the HA surface density: 1) by limiting the amount of proteolytically cleaved (and therefore activated) HA on the cell surface, and 2) by varying the surface density of fully activated HA. In both cases the low pH induced fusion delay time decreases with increasing densities of active HA.

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IDENTIFICATION OF POTENTIAL TYROSINE PHOSPHORYLATION SUBSTRATES IN T LYMPHOMA CELL MEMBRANES

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Tyrosine phosphorylation of specific proteins occurs after T cell activation with both antigen and interleukin-2. Several tyrosine phosphokinases (TPKs) and some of their substrates have been identified in T cells, but the functional relationships of TPKs with their substrates have yet to be clarified in T cell activation pathways. In particular, membrane-associated TPK substrates involved in the generation of second messengers still need to be characterized.
To this end, plasma membrane fractions were obtained from constitutively activated T lymphoma cells and their proteins analyzed by two-dimensional electrophoresis. Total phosphoproteins (a) and tyrosyl-phosphoproteins (b) were labelled *in vitro* with γ-^{32}P-ATP (a), or detected by 2-D immunoblotting using anti-phosphotyrosine antibodies (b). Comparison of the total proteins, total phosphoprotein and phosphotyrosylprotein maps indicate that only select and minor membrane proteins are phosphorylated under the conditions used. Moreover, tyrosine phosphorylated substrates constitute a further subpopulation of 8-10 phosphoproteins that probably reflect the specificity of endogenous membrane TPKs.

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INTERACTIONS BETWEEN A SURFACE PROTEIN AND A POTENTIAL TRANSCRIPTION FACTOR AFFECTING THE GROWTH PATTERN IN *DROSOPHILA*

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We present data concerning the identification as well as genetic and molecular characterization of the genes *aristaless* (*al*) and *dachsous* (*ds*) in *Drosophila*. The *al* mutation reduces or deletes the most distal antennal segment and diminuishes the size of the scutellum. The *ds* gene affects the morphogenesis of legs and wings. We have found that two different, genetically interacting genes cause the *ds* phenotype. In collaboration with H. Clark and C. Goodmann (UC Berkeley), we could identify one gene as a potential cadherin. In vertebrates, these integral membrane proteins promote cell aggregation in a Ca^{2+}-dependent manner. The other *ds* gene contains a *prd*-type homeobox and hence probably is a transcription factor. This locus is also responsible for the *al* phenotype when mutated as in the *In(2L)al*^{130}.

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ACTIVATION OF PROTEIN KINASE C INCREASES PHOSPHOLIPASE A2 ACTIVITY AND PROSTACYCLIN PRODUCTION IN RAT CARDIOMYOCYTES

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A number of stimuli inducing increased phospholipase A2 (PLA2) activity and eicosanoid production also activate the phospholipid and calcium dependent protein kinase C (e.g. tumor promoters, hormones, neurotransmitters). In order to investigate the possible relationship between these concomitant increases in enzymatic activity, cultured rat cardiomyocytes were stimulated with the known protein kinase C activator phorbol 12-myristate 13-acetate and assayed for both PKC and PLA2 activity, as well as for prostacyclin (PGI2) production. At a concentration of 0.1 μM, PMA induced: a) a rapid increase in particulate bound PKC activity (+25% ±6.1% at 15 min, n = 5) accompanied by a corresponding decrease of activity in the cytosol (-60% ± 12.2%, n = 7), b) the activation of phospholipase A2 (+65% ± 15.2% at 15 min, n = 3) and c) an important increase in PGI2 production (+ 302% ± 74%, n = 10). This PMA induced increase could be modulated in a concentration-dependent manner (EC50 ≈ 0.01 μM). Conversely, the PKC inhibitor staurosporin (1 μM) inhibited basal PGI2 production by 51% (± 12.3%, n = 6) and totally abolished PMA induced PGI2 production. The inactive phorbol ester analogues, 4a-phorbol and phorbol 12-monoacetate exhibited no effect. Taken together, these results indicate that cardiac PLA2 activity and eicosanoid production is modulated by PKC.

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SIGNAL TRANSDUCTION VIA PROTEIN KINASE C MODULATES RADIATION INDUCED G2 DELAY

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The physiological basis for the delayed entry into mitosis of eukaryotic cells after exposure to ionizing radiation is unknown. Because caffeine inhibits G2 delay, studies with both cyclic AMP and calcium ions have been conducted but these were found to have no effect. Using flow cytometry we investigated the effects of the phorbol ester, PMA, and ionomycin on V79 Chinese hamster fibroblasts. These compounds influence protein kinase C activity, which is the central step in the phospholipase C signal pathway. Delayed entry into mitosis was determined from temporal perturbations of the cell cycle distribution. PMA induced a partial G2 delay which was further enhanced by ionomycin. The G2 delay produced by the combined treatment was similar to that by radiation but was not responsive to caffeine. The results suggest that protein kinases mediate the delay and that caffeine acts early in the G2 delay signal pathway.

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EXPRESSION OF PROTEIN KINASES DURING *in vitro* NEUROBLASTOMA DIFFERENTIATION.

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Protein kinases are being recognized as an interconnected signalling network and they are involved both in the ontogeny and in specialized functions of neuronal tissues. Transcriptional control of one of these genes during differentiation could be a clue to its decisive role. As a model for neuronal differentiation we use human neuroblastomas treated for 48 hours with the natural morphogen retinoic acid. cDNA fragments are cloned using polymerase chain reaction and degenerate primers to conserved sequences of the kinase domain. The cDNAs are screened for differential mRNA levels in control- and retinoic acid-induced cells. After preliminary screening for such regulated cDNAs promising candidates are identified by nucleic acid sequencing and further characterized by Northern blot and RNase protection analysis. Currently, we are exploring the possibilities of substraction procedures using the polymerase chain reaction.

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HIGH-LEVEL EXPRESSION OF WILDTYPE AND PHE102TRP RAT PARVALBUMIN IN E. COLI

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Parvalbumin (PV) is a small cytosolic protein with three EF-hand type helix-loop-helix motifs known to be responsible for high affinity Ca^{2+}-binding. In order to study Ca^{2+}-induced conformational changes by fluorescence measurements we have cloned the rat PV cDNA into the expression vector pGEMEX-2 followed by site-directed mutagenesis of the third (EF) domain. Using PV full lenght cDNA as a template, a 700 bp fragment was amplified by PCR. The upstream primer contained a NdeI restriction site (CATATG) at the PV translation start codon. This site was used to clone the PV coding sequence into the expression vector at the ATG initiation codon of gene 10 from the T7 phage, which is controlled by the T7-polymerase promoter. High level expression was observed for E. coli strain JM 109 (DE3), resulting in a IPTG-inducible PV production of at least 30% with respect to total bacterial protein. In order to introduce a fluorescent label into PV, Phe at position 102 in the C-terminal helix of the EF-domain was replaced by a unique Trp using site-directed mutagenesis. This mutated PV should be a valuable tool in analyzing structural changes caused by Ca^{2+} uptake and release.