The inositol 1,4,5-trisphosphate receptor is essential for T-cell receptor signaling

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Communicated by Richard Axel, Columbia University, New York, NY, March 9, 1995

ABSTRACT Antigen-specific activation of T lymphocytes, via stimulation of the T-cell antigen receptor (TCR) complex, is marked by a rapid and sustained increase in the concentration of cytoplasmic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_c\)). It has been suggested that the second messenger inositol 1,4,5-trisphosphate (IP\(_3\)) produced after TCR stimulation binds to the IP\(_3\) receptor (IP\(_3\)R), an intracellular Ca\(^{2+}\)-release channel, and triggers the increase in [Ca\(^{2+}\)]\(_c\), that activates transcription of the gene for T-cell growth factor interleukin 2 (IL-2). However, the role of the IP\(_3\)R in T-cell signaling and possibly in plasma membrane Ca\(^{2+}\) influx in T cells remains unproven. Stable transfection of T cells (Jurkat) with antisense type 1 IP\(_3\)R cDNA prevented type 1 IP\(_3\)R expression, providing a tool for dissecting the role of IP\(_3\) signaling during T-cell activation. T cells lacking type 1 IP\(_3\)R failed to increase [Ca\(^{2+}\)]\(_c\), or produce IL-2 after TCR stimulation. Moreover, depletion of intracellular Ca\(^{2+}\) stores without TCR activation stimulated Ca\(^{2+}\) influx in cells lacking the type 1 IP\(_3\)R. These results establish that the type 1 IP\(_3\)R is required for intracellular Ca\(^{2+}\) release that triggers antigen-specific T-cell proliferation but not for plasma membrane Ca\(^{2+}\) influx.

Antigen-specific activation of T lymphocytes initiates a series of signal-transduction events fundamental to immune responses that protect the host against intracellular pathogens. T-lymphocyte activation, via the T-cell antigen receptor (TCR) requires an increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)), which is due to both a rapid transient release of intracellular Ca\(^{2+}\) and a subsequent prolonged influx of Ca\(^{2+}\) from the extracellular compartment (1–3). This increase in [Ca\(^{2+}\)]\(_c\) has been linked to T-cell responses, including the transcriptional activation of the gene for T-cell growth factor interleukin 2 (IL-2). However, direct evidence showing that the increase in [Ca\(^{2+}\)]\(_c\) is dependent on inositol 1,4,5-trisphosphate (IP\(_3\)) and the IP\(_3\) receptor (IP\(_3\)R) in T cells has been lacking.

An early event in the T-cell activation signaling pathway is phosphorylation of phospholipase C\(_\gamma\) by tyrosine kinases (4), resulting in production of the second messengers IP\(_3\) and diacylglycerol (DAG) (reviewed in ref. 5). DAG activates protein kinase C (PKC). IP\(_3\) binds to its receptor, an intracellular Ca\(^{2+}\)-release channel on the endoplasmic reticulum (ER) of human T lymphocytes (6), and presumably induces Ca\(^{2+}\) release that triggers plasma membrane Ca\(^{2+}\) influx and subsequent T-cell activation (7, 8).

Recently we cloned the 9.5-kb cDNA encoding the type 1 human T-lymphocyte IP\(_3\)R with a predicted molecular mass of 308 kDa (6). We used the most 5’ 2.9 kb of the human type 1 IP\(_3\)R antisense cDNA to stably transfect Jurkat (human lymphoma) cells and block expression of the IP\(_3\)R. In T cells lacking the IP\(_3\)R, no increase in [Ca\(^{2+}\)]\(_c\) was observed after stimulation of the TCR. However, transcription leading to expression of both early (CD69) and late (IL-2) markers for T-cell activation was intact when stimulated via non-TCR-dependent pathways. Moreover, capacitative Ca\(^{2+}\) entry, stimulated by depletion of intracellular stores, was also intact in cells lacking type 1 IP\(_3\)R.

METHODS Blocking Expression of the IP\(_3\)R in Jurkat Cells. The 5’ 2.9 kb (from nucleotide position –247 to 2592) of the gene for human type 1 IP\(_3\)R cDNA (designed ITPR1) (6) was cloned into the pREP10 vector in the antisense orientation and into the pREP7 vector in the sense orientation. Transfections of 5 µg of each plasmid into Jurkat cells were performed by using 5 µg of N-[(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate (DOTAP; Boehringer Mannheim) per ml. Stable transfectants were selected by using hygromycin (2 mg/ml). For all experiments, the following transfection controls were performed: (i) sense IP\(_3\)R DNA with an insert of the same 2.9-kb fragment of 5’ type 1 IP\(_3\)R and (ii) sense and antisense vectors with no insert. Jurkat cells were grown in RPMI 1640 medium containing 5% (vol/vol) fetal bovine serum (FBS), 100 units of penicillin per ml, and 100 µg of streptomycin per ml. Medium was changed every 48 hr. The anti-IP\(_3\)R antibody used in this experiment was raised against a synthetic peptide corresponding to the carboxyl-terminal 10 amino acids of the type 1 IP\(_3\)R. A second anti-IP\(_3\)R antibody raised against a synthetic peptide corresponding to amino acid residues 2652–2663 of the type 1 IP\(_3\)R gave identical results. Both antibodies were raised in rabbits as described (9). Permeabilized (100% methanol for 5 min at room temperature) or nonpermeabilized Jurkat cells were treated with affinity-purified anti-IP\(_3\)R antibody at 1:10,000 dilution. Cells were washed three times in phosphate-buffered saline (PBS) containing 2% FBS, followed by incubation with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC). Cells were washed and fixed in 1% paraformaldehyde. Three thousand cells were analyzed for each sample by using a FACS analyzer (Coulter). Cells incubated with either preimmune rabbit serum or secondary antibody alone served as negative controls. Staining Jurkat cells with an antibody preabsorbed with the antigenic peptide gave no signal above background, indicating the specificity of the antibody for the IP\(_3\)R. No signal was seen when nonpermeabilized Jurkat cells were stained with either anti-IP\(_3\)R antibody because the epitopes are intracellular (6).

[Ca\(^{2+}\)]\(_c\), Measurements. Jurkat cells in RPMI 1640 medium were added to coverslips coated with Cell-Tak (Biocoat; Sigma) and incubated for 20 min at 37°C. Then 5 µM fluo-3 acetoxyethyl ester (AM) (Molecular Probes) was added, and incubation was continued for 40 min at 37°C. The treated cells were diluted 1:9 in RPMI 1640 medium and incubated for an additional 30 min at 37°C. The cells were washed three times

Abbreviations: IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_3\)R, IP\(_3\) receptor; TCR, T-cell antigen receptor; [Ca\(^{2+}\)]\(_c\), concentration of cytoplasmic (intracellular) free Ca\(^{2+}\); ER, endoplasmic reticulum; FMA, phorbol 12-myristate 13-acetate; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; IL-2, interleukin 2.

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with Krebs–Ringer solution containing 1 mM Ca\(^{2+}\) or 40 \(\mu\)M Ca\(^{2+}\) (considered Ca\(^{2+}\) "free"). Coverslips were mounted into a microscope tissue chamber. Measurements of [Ca\(^{2+}\)]\(_i\) in Jurkat cells were performed with a Nikon inverted microscope. Data analysis was performed with KINEPHOTO software (Kinetek, Yonkers, NY). The cells were stimulated with an anti-CD3 monoclonal antibody (mAb) (clone 446; 1:20 dilution of a cultured supernatant). At the conclusion of each trial, maximal fluorescence (\(F_{\text{max}}\)) was obtained by addition of 6 \(\mu\)M digitonin and 5 mM CaCl\(_2\). Minimal fluorescence was obtained by exchanging the Ca\(^{2+}\) solution for 6 \(\mu\)M digitonin with 20 mM EGTA. [Ca\(^{2+}\)]\(_i\) was calculated from these values by using the equation: 
\[
[\text{Ca}^{2+}]_i = KA(F - F_{\text{min}})/(F_{\text{max}} - F),
\]
where \(K_A\) is 400 nM (10). Fluorescence measurements were performed at room temperature.

**Determinations of IL-2 Activity and CD69 Levels.** Transfected Jurkat cells (10\(^5\) cells) were cultured in 96-well plates coated with anti-CD3 antibody. Supernatants were harvested after 20 hr of culture, and IL-2 activity was determined by using CTLL-2 cells, and IL-2 activity was calculated with recombinant IL-2 as a standard. CD69 antigen was determined by flow cytometry with an FITC-conjugated mouse anti-human CD69 mAb (clone FN50; PharMingen) after activation with (i) anti-CD3 (0.25−2.0 \(\mu\)g/ml; PharMingen) or (ii) with phorbol 12-myristate 13-acetate (PMA; Sigma; 10 ng/ml) and ionomycin (500 nm/ml), or (iii) PMA (10 ng/ml) and anti-CD28 antibody (clone NE51; 1:2 to 1:8 dilution of a cultured supernatant). Stable transfectants (0.5 × 10\(^6\) cells per ml) were cultured in plates coated with anti-CD3 mAb for 12 hr followed by staining with anti-CD69 mAb conjugated to FITC.

**RESULTS AND DISCUSSION**

An anti-IP\(_3\)R antibody detected IP\(_3\)R protein in wild-type Jurkat cells (Fig. 1 Left Upper) but not in antisense cDNA-transfected Jurkat cells (Fig. 1 Left Lower). In these experiments an anti-IP\(_3\)R peptide antibody that only recognizes type 1 IP\(_3\)R was used. Identical results were obtained with a second anti-IP\(_3\)R antibody that recognizes an epitope 90% identical to the type 2 IP\(_3\)R and 70% identical to the type 3 IP\(_3\)R. Experiments with an anti-CD3 mAb that recognizes the TCR–CD3 complex showed that both wild-type and IP\(_3\)R antisense transfectants expressed normal levels of TCR (Fig. 1 Right).

Activation of the TCR in wild-type Jurkat cells with the anti-CD3 mAb resulted in a rapid increase in [Ca\(^{2+}\)]\(_i\) due to both an initial intracellular Ca\(^{2+}\) release followed by a sustained Ca\(^{2+}\) influx (\(n = 16\), Fig. 2 Upper Left). The initial, rapid increase in [Ca\(^{2+}\)]\(_i\), but not the subsequent sustained Ca\(^{2+}\) influx, was present in Ca\(^{2+}\)-free solution (\(n = 9\); e.g., Fig. 2 Upper Right). In Jurkat cells lacking the IP\(_3\)R, there was neither release of intracellular Ca\(^{2+}\) nor Ca\(^{2+}\) influx in response to anti-CD3 (\(n = 20\), Fig. 2 Lower Left). This lack of Ca\(^{2+}\) flux was specifically due to transfection with IP\(_3\)R antisense cDNA because the sense construct corresponding to this same IP\(_3\)R sequence had no effect on intracellular Ca\(^{2+}\) release (\(n = 7\)), nor did either sense (\(n = 4\)) or antisense vectors (\(n = 9\)). These data indicate that the IP\(_3\)R is essential for anti-CD3 mAb-activated intracellular Ca\(^{2+}\) release and for the subsequent Ca\(^{2+}\) influx.

To determine whether the IP\(_3\)R itself was the channel for plasma membrane Ca\(^{2+}\) influx in T cells or whether depletion of intracellular stores independent of the IP\(_3\)R, was sufficient to trigger Ca\(^{2+}\) influx, we used thapsigargin to deplete the intracellular stores in Jurkat cells that lack the IP\(_3\)R. Thapsigargin is an inhibitor of the ER Ca\(^{2+}\)-ATPase and has been shown to trigger Ca\(^{2+}\) influx across the plasma membrane in the absence of TCR activation (11). Thapsigargin does not stimulate IP\(_3\) production (12). In IP\(_3\)R antisense cDNA-transfected T cells, thapsigargin released intracellular Ca\(^{2+}\), demonstrating that the ER pool of Ca\(^{2+}\) was intact (Fig. 2 Lower Right). In cells that lacked the IP\(_3\)R, depletion of intracellular Ca\(^{2+}\) stores with thapsigargin-activated the influx of Ca\(^{2+}\) across the plasma membrane (Fig. 2 Lower Right). This Ca\(^{2+}\) influx was blocked by addition of 5 mM nickel, an
inhibitor of plasma membrane Ca\(^{2+}\) influx (data not shown). This result shows (i) that the lack of Ca\(^{2+}\) influx during T-cell activation in the IP\(_3\)R antisense cDNA transfectants was due to inability to deplete IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores because of the lack of the IP\(_3\)R, and (ii) that the IP\(_3\)R is not the plasma membrane Ca\(^{2+}\) influx channel in T cells.

Our current data support the capacitative model for Ca\(^{2+}\) entry in nonexcitable cells in which Ca\(^{2+}\) influx across the plasma membrane is coupled to depletion of intracellular stores (13, 14). The signal connecting the depletion of intracellular Ca\(^{2+}\) stores to plasma membrane Ca\(^{2+}\) influx has not been established. It has also been proposed that IP\(_3\) is the second messenger that directly stimulates Ca\(^{2+}\) influx across the plasma membrane, possibly via the IP\(_3\)R itself (15, 16) or via a structurally unrelated Ca\(^{2+}\) channel in the plasma membrane (17). The present study shows that the type 1 IP\(_3\)R is not the plasma membrane Ca\(^{2+}\) influx channel during T-cell activation. Moreover, it has been suggested that a conformational change in the IP\(_3\)R, induced by emptying of the ER Ca\(^{2+}\) pool, may lead to the opening of a plasma membrane Ca\(^{2+}\) channel (18). Our data show that capacitative Ca\(^{2+}\) entry is intact in cells lacking the type 1 IP\(_3\)R and argue against a role for the type 1 IP\(_3\)R in communicating between the ER and the plasma membrane as proposed in the "conformational coupling hypothesis" (19). Two recent studies have suggested that a diffusible second messenger is involved in triggering plasma membrane Ca\(^{2+}\) influx (20, 21). The present data indicate that the type 1 IP\(_3\)R is not the target for such a second messenger.

In T cells, the prolonged increase in [Ca\(^{2+}\)]\(_i\) is necessary for IL-2 production. The increase in [Ca\(^{2+}\)]\(_i\) activates calcineurin, a Ca\(^{2+}\)/calmodulin-dependent phosphatase that dephosphorylates the cytoplasmic subunit of the transcription factor NF-AT, allowing it to translocate to the nucleus (22). In the nucleus the cytoplasmic subunit of NF-AT associates with the nuclear subunit (Fos and Jun) and activates transcription of the gene for the T-cell growth factor IL-2. IL-2 production, therefore, is Ca\(^{2+}\) dependent and is required for T-cell proliferation. In IP\(_3\)R antisense cDNA-transfected T cells, no IL-2 production was detected after TCR activation with anti-CD3 mAb (0.25–2.0 \(\mu\)g/ml) (e.g., Fig. 3 Left). IP\(_3\)R sense transfectants produced normal levels of IL-2 (data not shown). Ionomycin (a Ca\(^{2+}\) ionophore) plus PMA (an activator of PKC), a combination that activates T cells by bypassing the TCR (1, 23), induced normal levels of IL-2 production (Fig. 3 Left) in both control and in IP\(_3\)R antisense transfectants. PMA with anti-CD28 antibody activates T lymphocytes via a TCR-independent pathway (24, 25). In antisense IP\(_3\)R transfected, PMA combined with anti-CD28 antibody resulted in the same level of IL-2 production as in control wild-type cells (Fig. 3 Left). This result indicated that the IP\(_3\)R-independent components of the T-cell activation pathway were intact in the IP\(_3\)R antisense cDNA transfectants.

Jurkat cells lacking IP\(_3\)R exhibited an \(\approx\)75% decrease in the rate of serum-stimulated cell growth (Fig. 3 Right); however, cell viability was not altered. The finding that the lack of the IP\(_3\)R slows cell growth supports the hypothesis that IP\(_3\)R-mediated Ca\(^{2+}\) signaling may play a role in regulating cell growth and in T-cell activation (19). However, unlike antigen-specific T-cell activation via the TCR, which was completely dependent on the IP\(_3\)R, lack of the IP\(_3\)R only partially inhibited cell growth. This finding suggests that signal transduction for serum-stimulated cell growth occurs via more than one sig-

![Fig. 2.](image-url)

**Fig. 2.** Activation of the TCR does not mobilize Ca\(^{2+}\) in fluo-3-loaded T cells that lack IP\(_3\)R. (Upper Left) Addition of soluble anti-CD3 mAb (αCD3) elicited a rapid and sustained increase in [Ca\(^{2+}\)]\(_i\) in control Jurkat cells (with 1 mM extracellular Ca\(^{2+}\)). (Upper Right) In the absence of extracellular Ca\(^{2+}\), anti-CD3 mAb induced the release of intracellular Ca\(^{2+}\). Addition of 500 nM thapsigargin (Tg) triggered a second, smaller release of intracellular Ca\(^{2+}\). Subsequent addition of 1 mM extracellular Ca\(^{2+}\) resulted in a large Ca\(^{2+}\) influx across the plasma membrane. (Lower Left) Anti-CD3 antibody failed to induce an increase in [Ca\(^{2+}\)]\(_i\) in Jurkat cells lacking IP\(_3\)R in the presence of 1 mM extracellular Ca\(^{2+}\). (Lower Right) Again, application of anti-CD3 mAb did not release intracellular Ca\(^{2+}\) (in the absence of extracellular Ca\(^{2+}\)). Subsequent addition of 500 nM thapsigargin elicited a rapid release of intracellular Ca\(^{2+}\) in IP\(_3\)R antisense cDNA-transfected Jurkat cells (demonstrating that the intracellular Ca\(^{2+}\) pool was intact). Subsequent addition of extracellular Ca\(^{2+}\) increased [Ca\(^{2+}\)]\(_i\) in the absence of the IP\(_3\)R, indicating that the IP\(_3\)R is not required for plasma membrane Ca\(^{2+}\) influx. The bottom dotted line (unlabeled arrow) shows the effect of adding extracellular Ca\(^{2+}\) without prior addition of thapsigargin in T cells lacking IP\(_3\)R. Tracings are representative data obtained from single cells; each experiment was repeated multiple times (see text).

FIG. 3. (Left) T lymphocytes that lack the IP3R receptor do not produce IL-2 after TCR stimulation. IL-2 production stimulated by cross-linking with anti-CD3 mAb (αCD3) is blocked in Jurkat cells lacking IP3R. Control Jurkat cells secreted IL-2 after activation with anti-CD3 mAb or with PMA and ionomycin or with PMA and anti-CD28 antibody (αCD28). IP3R antisense cDNA-transfected cells produced no IL-2 after activation with anti-CD3 mAb, whereas PMA and ionomycin or PMA and anti-CD28 antibody induced levels of IL-2 comparable to those seen in the control (wild-type) cells. (Right) T lymphocytes lacking the IP3R have reduced serum-stimulated rates of growth. Cell growth was slowed in T cells (Jurkat) lacking IP3R. Cell counts were determined at the indicated time points after addition of 10% fetal calf serum. Cell viability assessed by trypan blue exclusion was the same for wild type and antisense cDNA transfectants. Each time point represents the average of three experiments; error bars represent SEM.

... signaling pathway, whereas antigen-specific T-cell activation is solely mediated by the IP3 signaling pathway. It has been shown that the initial early increase in [Ca2+]i is not by itself sufficient to stimulate gene expression leading to IL-2 production (26, 27). The lack of IL-2 production in cells that do not express IP3R is due to a failure to generate a prolonged increase in [Ca2+]i after activation by anti-CD3 mAb. This inability to trigger the Ca2+ influx required to sustain a prolonged increase in [Ca2+]i is directly due to the lack of the early release of intracellular Ca2+ via the IP3R after TCR stimulation and not to a defect in the plasma membrane Ca2+ influx pathway, which remains intact in IP3R antisense cDNA transfectants (Fig. 2 Lower Right). CD69 is an early lymphocyte activation marker induced by anti-CD3 antibody.

FIG. 4. Detection of CD69 antigen (light trace), an early marker of T-cell activation. T cells (Jurkat) were activated by the indicated stimuli, stained with anti-CD69 mAb and analyzed. (A) Wild-type Jurkat cells (control) (light trace) exhibited CD69 antigen 12 hr after stimulation with anti-CD3 mAb (αCD3) compared with unstimulated cells (control). Unstimulated cells did not express CD69, as expected. (B) Jurkat cells lacking IP3R showed no detectable CD69 antigen expression after stimulation with anti-CD3 mAb as compared to unstimulated cells (control). (C) Activation of wild-type cells with PMA and ionomycin resulted in CD69 expression. (D) IP3R antisense cDNA-transfected T cells activated with PMA and ionomycin resulted in levels of CD69 expression that were comparable to those seen in wild-type cells. (E) Wild-type Jurkat cells (control) activated with PMA and anti-CD28 antibody (αCD28) induced CD69 expression as did Jurkat cells lacking IP3R (F). Each experiment measuring CD69 expression was repeated at least three times with similar results.
(28, 29). CD69 was detected 12 hr after activation with anti-CD3 mAb in control cells (Fig. 4A). No detectable CD69 was present in IP3R antisense cDNA-transfected cells (Fig. 4B). Activation of IP3R antisense cDNA-transfected T cells with PMA and ionomycin resulted in CD69 expression comparable to that seen in wild-type cells (Fig. 4 C and D). PMA with anti-CD28 mAb induced CD69 expression to the same degree in control wild-type cells (Fig. 4E) and in IP3R antisense cDNA transfectants (Fig. 4F). Taken together, these findings show that the IP3R is absolutely required for TCR-mediated responses as determined by expression of both early (CD69) and late (IL-2) markers of T-lymphocyte activation. Moreover, the Ca2+-independent pathways for T-cell activation were intact in the IP3R antisense cDNA transfectants.

Two other forms of IP3R have been identified. The type 2 IP3R has been cloned from rat cerebellum (30) and human endothelial cells (31) and shares 69% amino acid identity with the type 1 IP3R. The type 3 IP3R shares 64% identity with the amino acid sequence of the type 1 IP3R (31–33). This IP3R antisense cDNA is sufficiently homologous to the type 2 IP3R for it to block expression of both types 1 and 2 IP3R. However, the homology with the type 3 IP3R in this region of the IP3R cDNA was not significant. The IP3R/intracellular Ca2+ release channel is thought to be formed by a tetramer of four IP3Rs (34). Because the IP3R structure is a tetramer, it is possible that the functional channel could comprise a mixture of types 1, 2 and 3; however, our demonstration that cells in which no type 1 IP3R is detected cannot be activated or mobilize Ca2+ implies that the types 2 and 3 IP3R, if present, cannot replace the essential function of the type 1 IP3R in antigen-specific activation of T cells.

The present study shows a specific and highly specialized function for the IP3R in a cell—that is, mediating the increase in [Ca2+]i required for antigen-specific T-cell activation. Many cell types contain more than one form of intracellular Ca2+ release channel, and another form of intracellular Ca2+ release channel—the ryanodine receptor—has been reported in T cells (35). With the exception of skeletal muscle, where a knock-out of the ryanodine receptor prevented excitation-contraction coupling (36), lack of expression of a member of the intracellular Ca2+ release channel family has not previously been reported in any cell type to our knowledge. In Jurkat cells, absence of the IP3R specifically abrogates antigen-specific T-cell activation but only partially inhibits serum-stimulated cell growth, suggesting that growth factors that bypass the TCR are not critically dependent on the IP3R for signal transduction. The specificity of IP3R function during T-cell activation signaling suggests that this Ca2+ channel could be a useful pharmacologic target for modulating the immune response.

We thank Drs. Jay Unkeless, Lloyd Mayer, Wayne Yokoyama, and Barbara Ehrlich for helpful comments on this manuscript. The antibody recognizing the carboxyl-terminal 10 amino acids of type 1 IP3R was provided by Dr. Barbara Ehrlich. We thank Dr. Lloyd Mayer for the anti-CD28 antibody, Jurkat cells, and CTLT cells. This work was supported by grants to A.R.M. from the National Institutes of Health (NS29814) and the American Heart Association. A.R.M. is a Bristol-Myers Squibb Established Investigator of the American Heart Association. D.J.H. is a Howard Hughes Medical Student Fellow. K.O. is supported by a grant from the Louis B. Mayer Foundation; K.O. and E.O. are on leave from the Institute of Experimental Pharmacology, Bratislava, Slovakia.