# EXTRACELLULAR ATP AND $Hg^{2+}$ MOBILIZE $Ca^{2+}$ FROM DISTINCT INTRACELLULAR POOLS IN HEPATOCYTES ISOLATED FROM THE LITTLE SKATE RAJA ERINACEA

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We have previously shown that skate hepatocytes contain ATP receptors, and that stimulation of these receptors increases cytosolic $Ca^{2+}$ ($Ca_i^{2+}$)(M.H. Nathanson and K. Mariwalla, Amer. J. Physiol. (in press), 1996). This $Ca_i^{2+}$ increase consists of two components: an early phase due to release of internal $Ca^{2+}$ stores, which reaches its peak within seconds, and a late, prolonged phase due to influx of extracellular $Ca^{2+}$, which persists for minutes. We also have shown that $Hg^{2+}$ increases $Ca_i^{2+}$, although this $Ca_i^{2+}$ increase is dose-dependent and predominantly due to release of $Ca^{2+}$ from internal stores (M.H. Nathanson et al, Cell Calcium 18:429-439, 1995). The purpose of this study was to further characterize the receptors and internal stores responsible for ATP-induced $Ca_i^{2+}$ signals, and to determine the relationship between these $Ca^{2+}$ stores and those mobilized by $Hg^{2+}$. Hepatocytes were isolated by collagenase perfusion (D.J. Smith et al, Amer. J. Physiol. 252:G479-G484, 1987), then either loaded with the $Ca^{2+}$-sensitive dye indo-1 (10 μM) and examined by ratio spectrofluorometry (G. Grynkiewicz et al, J. Biol. Chem. 260:3440-3450, 1985) using a Perkin-Elmer LS-5B spectrometer, or loaded with the mitochondrial dye rhodamine-123 (5 μg/ml) and examined by confocal fluorescence microscopy.

Since there is variability in the response of skate hepatocytes to ATP, we first examined whether this variability was due in part to release of endogenous ATP, leading to desensitization of purinoceptors. We compared the response of control hepatocytes to hepatocytes pre-incubated with the ATP/ADPase apyrase. In control hepatocytes, the $K_m$ for ATP was 1.2±1.0 μM (mean±SEM), and in hepatocytes pre-incubated with apyrase the $K_m$ was not significantly different (1.6±1.1 μM); each $K_m$ was estimated by nonlinear regression from dose-response curves in which each data point was measured in duplicate or triplicate. Thus, apyrase did not alter the dose-response relationship for ATP, suggesting that desensitization of receptors due to release of endogenous nucleotides does not contribute to the variable response we observed. We also performed cross-desensitization studies to further characterize skate hepatocyte ATP receptors. Cells were stimulated sequentially with UTP (100 μM) followed by 2MeSATP (100 μM), or else by 2MeSATP followed by UTP. Stimulation with UTP initially increased $Ca_i^{2+}$ by 415±85 nM, and subsequent exposure to 2MeSATP further increased $Ca_i^{2+}$ by only 83±90 nM (n=3; $p<0.02$ by paired t test). In contrast, initial stimulation with 2MeSATP increased $Ca_i^{2+}$ by 329±18 nM, while subsequent exposure to UTP further increased $Ca_i^{2+}$ by 537±103 nM (n=3; $p>0.05$ by paired t test). Thus, UTP desensitized hepatocytes to 2MeSATP, while 2MeSATP did not similarly affect the response to UTP.

To investigate the relationship between internal $Ca^{2+}$ stores mobilized by ATP and $Hg^{2+}$, cells were placed in $Ca^{2+}$-free medium, then sequentially stimulated with ATP (100 μM) to mobilize agonist-sensitive $Ca^{2+}$ stores, the $Ca^{2+}$-ATPase inhibitor thapsigargin (5 μM) to further deplete these stores, and $Hg^{2+}$ (50 μM). Each of these additions increased $Ca_i^{2+}$ ($p<0.0001$, $p<0.02$, and $p<0.00005$, respectively). These $Ca_i^{2+}$ increases also occurred if thapsigargin was added before ATP (n=4). This suggests that ATP and $Hg^{2+}$ mobilize $Ca^{2+}$ from distinct internal stores. Mitochondria provide a $Ca^{2+}$ pool that is not mobilized by agonists such as ATP. Since mitochondrial sequestration of $Ca^{2+}$ is dependent upon the maintainence of a highly negative mitochondrial membrane potential (T.E. Gunter et al, Amer. J. Physiol. 267:C313-C339, 1994), we examined the effect of $Hg^{2+}$ on this potential. Isolated skate hepatocytes were loaded with the cationic fluorescent dye rhodamine-123, which accumulates in

mitochondria in direct proportion to their membrane potential (A.L. Nieminen et al, J. Biol. Chem. 265:2399-2408, 1990). Mitochondrial fluorescence over time was measured by time-lapse confocal microscopy in the absence or presence of $Hg^{2+}$ (Figure 1). $Hg^{2+}$ accelerated the loss of fluorescence from mitochondria, suggesting that $Hg^{2+}$ decreases the potential gradient, permitting mitochondrial $Ca^{2+}$ to leak into the cytosol.

In summary, these findings suggest: (1) skate hepatocytes express ATP receptors which exhibit broad specificity, (2) stimulation of ATP receptors and application of $Hg^{2+}$ mobilize $Ca^{2+}$ from distinct internal stores, and (3) $Hg^{2+}$ dissipates the potential gradient of mitochondria, suggesting that mitochondria are the source of $Hg^{2+}$-induced $Ca_i^{2+}$ signals in this cell type. Additional work will be needed to demonstrate directly that depolarization induces $Ca^{2+}$ release from mitochondria, and to define the role of mitochondrial depolarization in $Hg^{2+}$-induced toxicity in skate hepatocytes.

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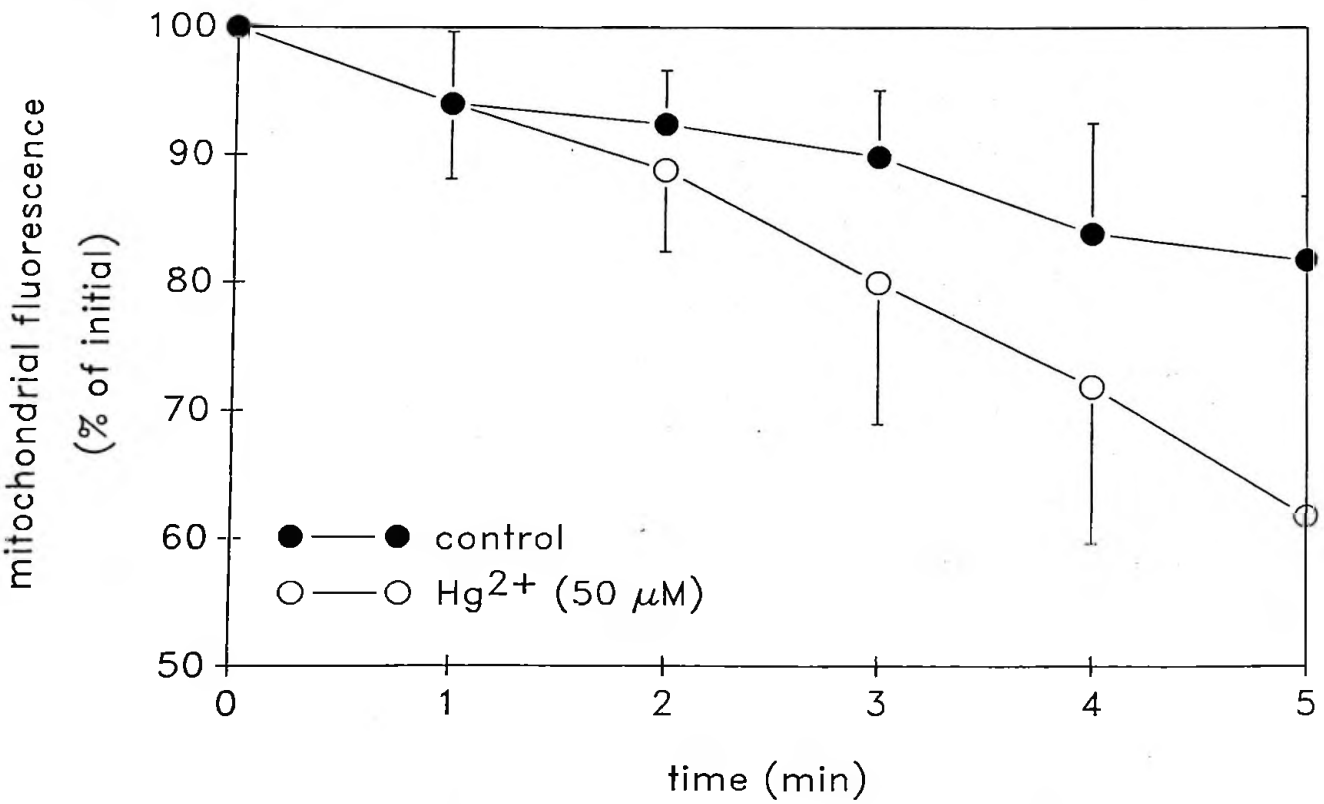


Figure 1. $Hg^{2+}$ decreases mitochondrial membrane potential in isolated skate hepatocytes. Hepatocytes were loaded with the fluorescent dye rhodamine-123, which accumulates in mitochondria in proportion to mitochondrial membrane potential. Mitochondrial fluorescence decreases slowly under control conditions (solid circles), and rapidly in the presence of $Hg^{2+}$ (open circles).