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Immunolocalization of mitoK$_{ATP}$ subunits in human osteoblast-like cells

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1. ABSTRACT

The mitochondrial ATP-dependent K channel (mitoK$_{ATP}$) has been shown to play a role in cellular protection against apoptosis, or programmed cell death. This channel has been identified and characterized in a number of cell and tissue types but to date the possible existence of mitoK$_{ATP}$ in osteoblastic cells has not been investigated. The aim of this investigation was to establish whether the mitochondria of human osteosarcoma-derived osteoblasts (SaOS-2 cells) contain the putative mitoK$_{ATP}$ subunits Kir6.1 and Kir6.2. Ultrathin sections of SaOS-2 cells were prepared for transmission electron microscopy using an adaptation of the Tokuyasu method, and immunolabelled using goat anti-Kir6.1 or anti-Kir6.2 antisera as the primary label, and a 10nm colloidal gold-conjugated donkey anti-goat secondary antibody. The suitability of the antisera and the immunostaining protocol were confirmed by using a sample of rat cardiac muscle as a positive control. Ultrastructural analysis revealed that SaOS-2 cells contain Kir6.2 but not Kir6.1, and that Kir6.2 is present in the mitochondria, but in extremely low abundance. These findings suggest that human osteoblast-like cells might contain mitoK$_{ATP}$ channels in which Kir6.2 is the pore-forming subunit, although it appears that these channels are likely to be present in extremely low abundance.

2. INTRODUCTION

The mitochondrial ATP-dependent K^+ channel (mitoK$_{ATP}$) was first described by Inoue, who used an inside-out patch clamp method to detect a highly selective, small conductance K^+ channel in fused giant mitoplasts prepared from inner membranes of rat liver mitochondria (1). The channel was reversibly inhibited by ATP, and also blocked by 4-aminopyridine (a K^+ channel inhibitor) and glibenclamide (a blocker of ATP-dependent K^+ channels).

The presence of mitoK$_{ATP}$ has since been demonstrated in the mitochondrial inner membranes of a number of other cells and tissues, including rat heart, rat skeletal muscle, rat brain, rat kidney, mouse brain, mouse heart, bovine heart, human cardiac muscle, and human T-lymphocyte and keratinocyte cell lines (2-11).

Research into the properties of mitoK$_{ATP}$ has been stimulated by the possible role of the channel in cellular protection against ischemia-reperfusion injury. MitoK$_{ATP}$ activity has been implicated as part of the protective mechanism in ischemic preconditioning (IPC), and pharmacological mitoK$_{ATP}$ openers can be used to induce cardioprotection by simulating the endogenous preconditioning effect (12-14). Similar processes whereby increases in the permeability of the mitochondrial inner

membrane to K^+ lead to improved cellular tolerance to ischemia-reperfusion injury have also been observed in other tissues, including liver, gut, brain and kidney (15).

In addition to its function as an effecter of IPC, mitoK_{ATP} also protects cells against apoptosis by preserving mitochondrial integrity and preventing opening of the permeability transition pore (PTP) in the mitochondrial inner membrane (16-18). Pharmacological activation of mitoK_{ATP} with diazoxide, a potent mitoK_{ATP} opener, preserved mitochondrial integrity and suppressed apoptotic markers in both rat ventricular myocytes and rat cerebellar neurons subjected to oxidative stress (19,20). Iptakalim, another potassium channel opener, also protects rat astrocytes from apoptosis caused by MPP^+, a neurotoxin. Abolition of these protective affects by a specific mitoK_{ATP} blocker suggests that the anti-apoptotic mechanism involves opening of mitoK_{ATP} channels (21). Similarly, mitoK_{ATP} opening has also been shown to protect human keratinocytes against UV radiation-induced apoptosis (11).

Nitric oxide (NO), an endogenous signaling molecule, protects various cell types from apoptotic insult (22-26). Activation of mitoK_{ATP} has been implicated in this mechanism (27). Ockaili suggested that NO has a role in diazoxide-induced mitoK_{ATP} opening (28). This was corroborated by Sasaki who presented indirect evidence that NO selectively activates mitoK_{ATP} (29). NO-induced ROS generation in cardiomyocytes during IPC has been shown to occur via a mechanism involving opening of mitoK_{ATP} (30, 31). Recently, direct evidence has been presented confirming that cardiac mitoK_{ATP} is activated by exogenous NO (32).

In osteoblasts, low concentrations of NO have similarly been shown to protect cells from apoptotic insult, and this raises the possibility that mitoK_{ATP} may have a role in the regulation of osteoblast apoptosis (33, 34).

Furthermore, Akt (protein kinase B, PKB) inhibits osteoblast apoptosis, and also promotes osteoblast differentiation and function, which together result in increased bone mass. Akt is also known to have a role in cardioprotection, by mediating inhibition of cardiomyocyte apoptosis (35). There is evidence that Akt mediates the opening of cardiac mitoK_{ATP} and it is therefore possible that mitoK_{ATP} channels may also be involved in Akt-mediated inhibition of apoptosis in osteoblasts (36).

We hypothesize therefore that osteoblast mitochondria might contain mitoK_{ATP}, and that this channel may have a role in the regulation of osteoblast apoptosis. Here we use immunoelectron microscopy to investigate the expression of the K_{ATP} pore-forming subunits Kir6.1 and Kir6.2 in human osteoblast mitochondria.

3. MATERIALS AND METHODS

3.1. Cells and culture conditions

Human osteosarcoma-derived osteoblastic cells (SaOS-2; ATCC-LGC Promochem, Teddington, United Kingdom) were cultured in alpha modified Eagle's minimum essential medium (alphaMEM; Sigma-Aldrich Company Ltd, Poole, United Kingdom) supplemented with 10% fetal bovine serum (FBS) and 50U/mL penicillin / 50mcg/mL streptomycin (Invitrogen Ltd, Paisley, United Kingdom). The culture medium was replaced every two days. Cells were grown to approximately 90% confluence in $75cm^2$ cell culture flasks at 37 degrees Celsius in a humidified atmosphere of 5% CO_2 / 95% air and passaged as necessary.

It was considered desirable to obtain a sample of a cell or tissue type previously shown to contain the subunits of interest, to serve as a positive control for the immunoelectron microscopy work on SaOS-2 cells. Antibody-based methods have previously been used to demonstrate mitochondrial expression of Kir6.1 and Kir6.2 in rat cardiomyocytes [37-39], and therefore a sample of fixed rat cardiac muscle was used for this purpose.

3.2. Antibodies and probes

The primary antibodies were commercial goat polyclonal IgG that had been raised against Kir6.1 (C-16, R-14) or Kir6.2 (G-16, N-18) of human origin (Santa Cruz Biotechnology, Inc. California, USA). The secondary antibody was 10nm colloidal gold-conjugated donkey anti-goat IgG (Aurion, Wageningen, The Netherlands).

3.3. Immunoelectron microscopy

Specimens were prepared for immunogold labeling using an adaptation of the method described by Tokuyasu, as follows (40). Cells were fixed by gently adding 10mL of a solution of 4% formaldehyde and 0.2% glutaraldehyde in PBS to the medium in the $75cm^3$ cell culture flask. After incubation for 5min at room temperature the fixative was removed, the cells were washed with PBS, the flask was flooded with 4% formaldehyde / 0.2% glutaraldehyde in PBS and the cells were fixed for a further 2h at room temperature. The fixative was removed and the cells were rinsed twice with PBS. 10mL of 1% gelatin in PBS was then added to the flask and the cells were firmly scraped into the supernatant using a rubber cell scraper. The resulting cell suspension was transferred to a conical centrifuge tube and centrifuged at 1600 RPM for 5min. The supernatant was removed and the pellet was resuspended in 10% gelatin in PBS at 37ºC and further centrifuged at 5000 RPM for 5min. The tube was placed on ice to allow the gelatin to solidify and the end of the tube was sectioned to allow removal of a gelatin block containing the cell pellet. The cell-containing block was trimmed into $2mm^3$ cubes and these were infused in 2.3M sucrose solution overnight at 4 degrees Celsius, then mounted on specimen pins and frozen by immersion in liquid nitrogen (LN_2).

The rat cardiac muscle was fixed by perfusion of the excised hearts on a Langendorff apparatus with 4% formaldehyde / 0.2% glutaraldehyde, washed with PBS and cut into $2mm^3$ cubes. The cubes of tissue were infused in 2.3M sucrose, mounted and frozen using the same method as that described above for the SaOS-2 specimens.

Ultrathin (~80nm) cryosections were cut at -120 degrees Celsius on a Leica cryoultramicrotome, and

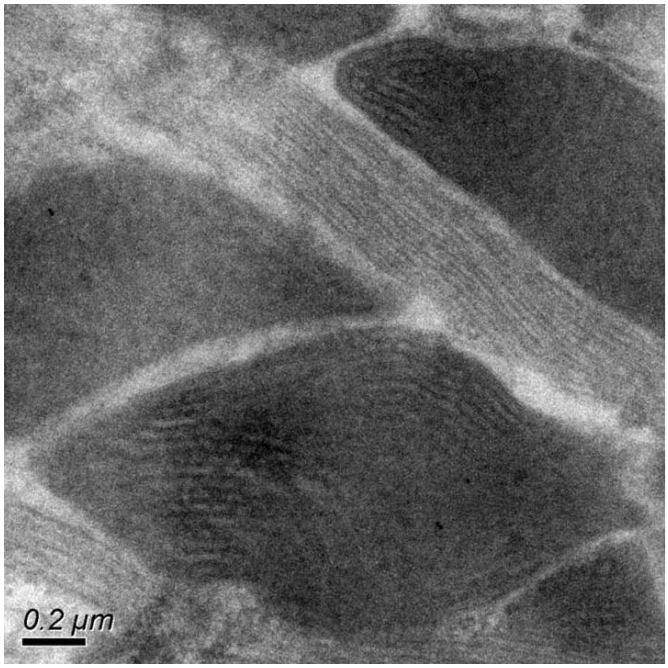

Figure 1. Immunoelectron micrograph of rat cardiomyocytes incubated with anti-Kir6.1 primary antibody. Colloidal gold particles are visible in the mitochondria, and no background labelling is evident.

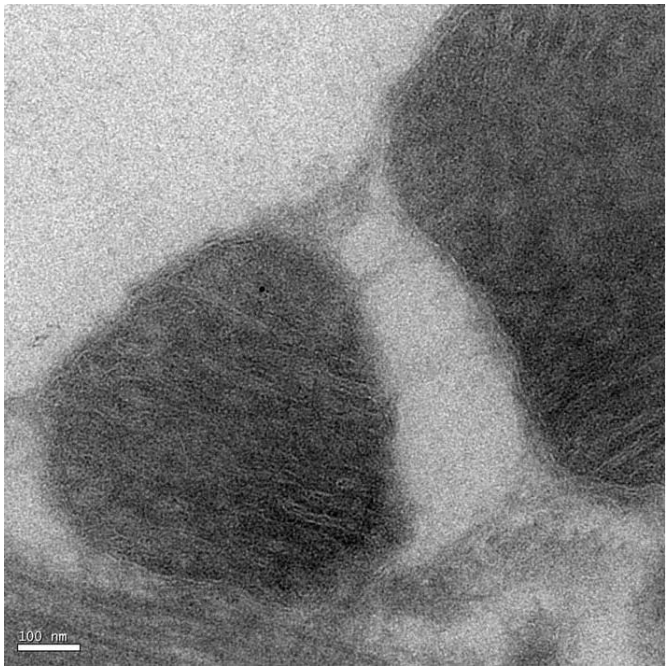

Figure 2. Higher magnification shows anti-Kir6.1 immunogold labelling localized to the mitochondrial inner membranes of rat cardiomyocytes.

transferred in droplets of 2.3M sucrose in a wire loop onto Formvar coated hexagonal mesh nickel grids that had been treated by glow discharge to reduce non-specific binding of gold particles (41). The grids were floated section side down onto PBS with 4% normal donkey serum with 0.1% azide (Sigma), at room temperature for 1h to reduce nonspecific binding of the secondary antibody, and then transferred over 3 droplets of 0.05M glycine in PBS with 1% BSA for 5 minutes per droplet, to quench any remaining aldehyde groups. After 3 washes over droplets

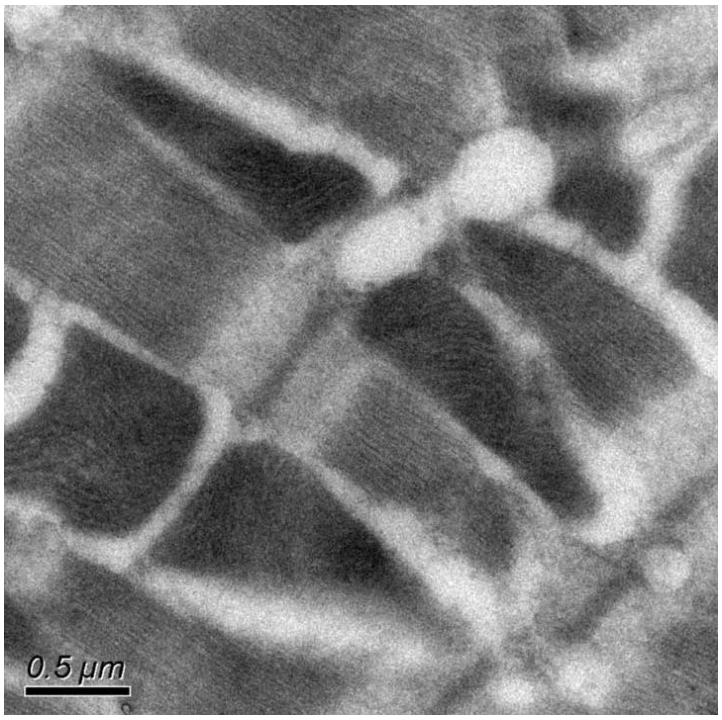

Figure 3. Immunoelectron micrograph of rat cardiomyocyte control section (primary antibody omitted), demonstrating a complete absence of labelling.

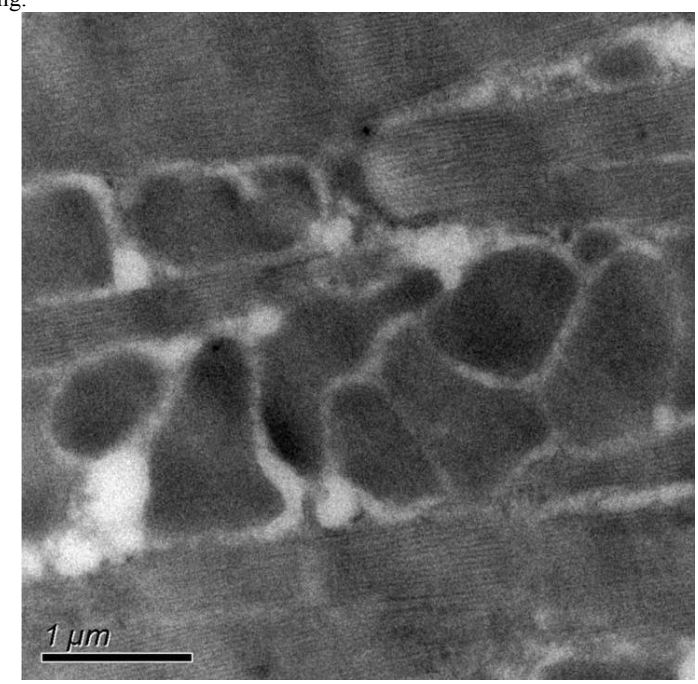

Figure 4. Rat cardiomyocyte control section. Numerous mitochondria are visible, lying alongside the myofibrils, and some t-tubules are also present. No gold particles are evident.

of PBS containing 0.1% acetylated BSA (BSA-c; Aurion) the sections were incubated with the primary antibodies at a dilution of 1:500 in PBS/BSA-c for 90 min in a humid chamber at room temperature. Some sections were incubated in the absence of the primary antibody to act as a control for non specific adherence of the secondary antibody. Overnight incubation with the primary antibodies at 4 degrees Celsius was also used, and gave similar results. Unbound primary antibody was removed by washing six times over PBS/BSA-c and the grids were

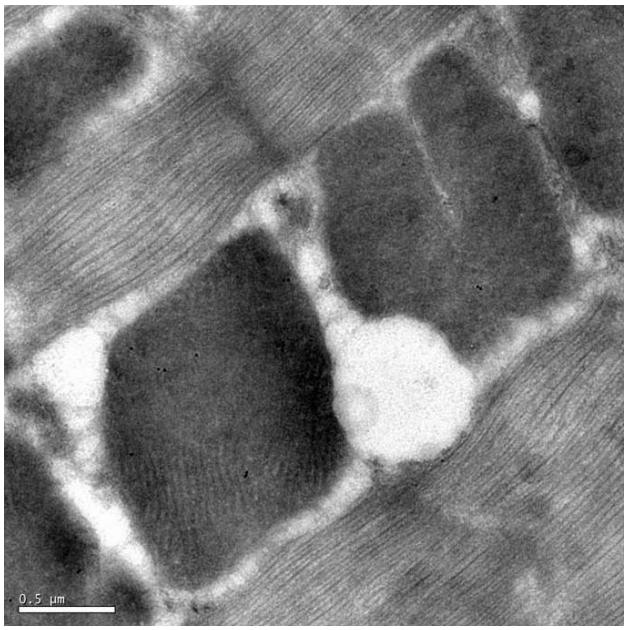

Figure 5. Immunoelectron micrograph showing immunogold labelling on a section of rat cardiomyocyte incubated with anti-Kir6.2 primary antibody. Specific labelling of the mitochondria can be seen, with no background label evident.

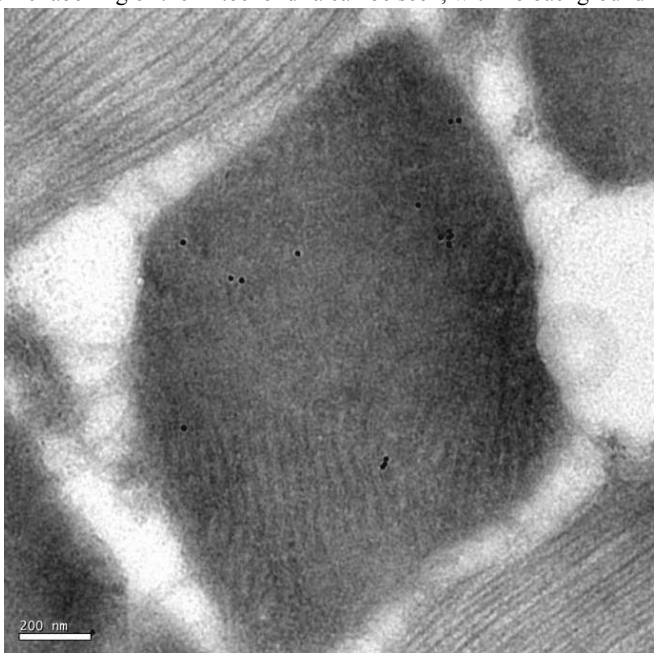

Figure 6. Higher magnification shows location of anti-Kir6.2 immunogold labelling on the mitochondrial inner membrane of rat cardiomyocyte.

then incubated with 10nm gold-conjugated donkey anti-goat secondary antibody at 1:40 dilution in PBS/BSA-c at room temperature for 1h. Following further washes with PBS/BSA-c and PBS, the immunoreaction was fixed by incubating with 2% glutaraldehyde for 5min. The specimens were washed several times with distilled water to remove the glutaraldehyde and any residual salts. The grids were placed onto drops of 4% uranyl oxalate for 10min, washed over distilled water and then placed onto a solution of 2% methylcellulose and 3% aqueous uranyl acetate (9:1) on ice for 10min to stain and embed the specimens. Excess methylcellulose was removed by blotting against hardened filter paper, and the grids were allowed to dry.

The specimens were examined in an FEI Tecnai T12 or T20 transmission electron microscope (FEI UK Ltd, Cambridge, United Kingdom) at accelerating voltages of

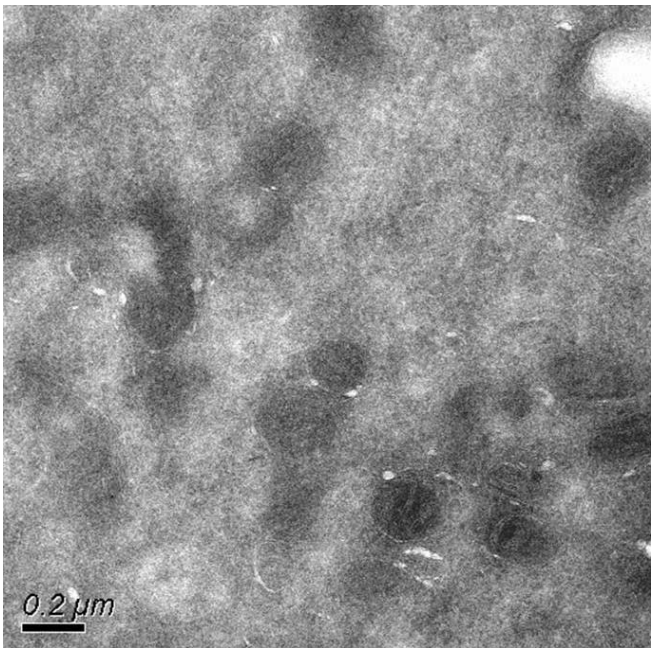

Figure 7. Immunoelectron micrograph showing a section of an SaOS-2 cell incubated with anti-Kir6.1 primary antibody. No specific mitochondrial labelling is evident, and background labelling is negligible.

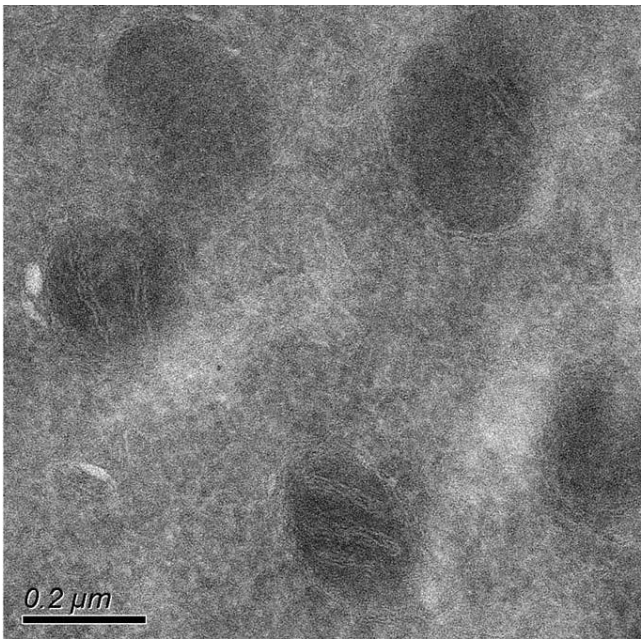

Figure 8. Another section of anti-Kir6.1 incubated SaOS-2 cell. Preservation of mitochondrial structure is evident, but no labelling is present.

120kV or 200kV respectively and imaged using Gatan digital cameras (Gatan UK, Oxfordshire, United Kingdom).

4. RESULTS

4.1. Rat cardiomyocytes

With the anti-Kir6.1 primary antibody, sparse labelling of the inner mitochondrial membranes, together with occasional labelling of the t-tubules, was observed using a 10nm gold-conjugated donkey anti-goat IgG secondary antibody (Figure 1, figure 2). Very little background gold label was observed, and the mitochondrial labelling was eliminated when the primary antibody was omitted from the control specimen (Figure 3, figure 4). This indicates that the observed distribution of gold on the

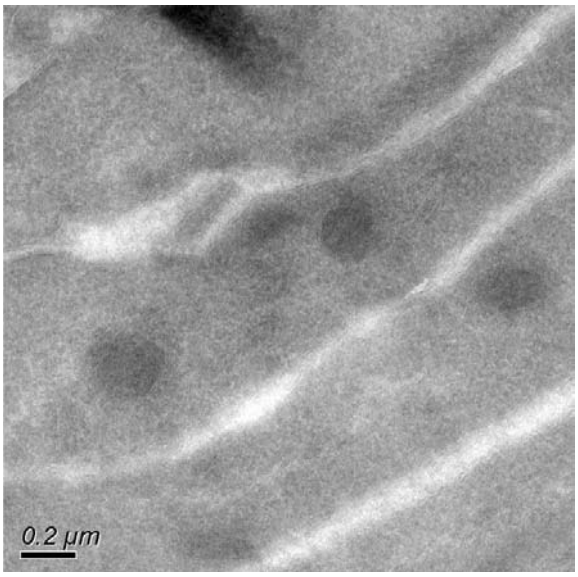

Figure 9. Control section of SaOS-2 cells (primary antibody omitted). No colloidal gold particles are visible.

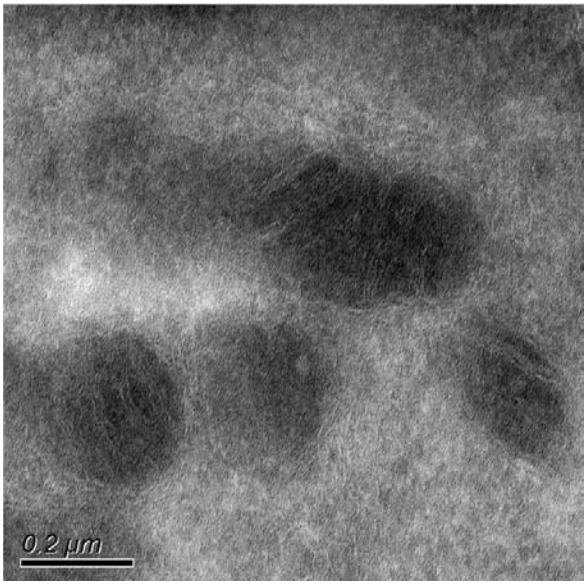

Figure 10. At higher magnification, good ultrastructural preservation is confirmed, and no labelling is evident.

experimental sections represents specific labelling of a membrane-bound protein by the anti-Kir6.1 antibody.

With the anti-Kir6.2 primary antibody and 10nm gold-conjugated donkey anti-goat secondary, a more intense specific mitochondrial labelling was observed, together with occasional particles on the t-tubules (Figure 5). At higher magnification, the gold labelling was situated on the mitochondrial inner membrane (figure 6). There was a general absence of background labelling, and almost no gold particles were observed on the control specimens. These findings confirm that the anti-Kir6.2 antibody specifically binds to a protein in the mitochondrial inner membrane of rat cardiomyocytes.

The results obtained from immunogold labelling of rat cardiomyocytes with anti-Kir6.1 and anti-Kir6.2 antisera verifies the suitability of the antibodies for this purpose, and was used as a positive control for the immunolabelling experiments using human osteoblast-like (SaOS-2) cells.

4.2. SaOS-2 cells

No specific anti-Kir6.1 gold labelling was observed on grids containing sections of SaOS-2 cells (Figure 7, figure 8). Very little background labelling was evident, similar to the control sections from which the primary antibody had been omitted (Figure 9, figure 10).

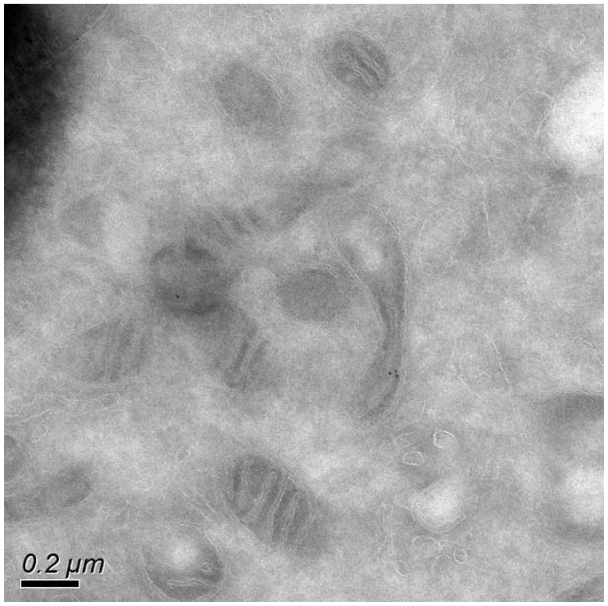

Figure 11. Immunogold electron micrograph showing anti-Kir6.2 labelling of SaOS-2 cell. Small amounts of specific mitochondrial labelling are evident. Labelling is present in relatively few mitochondria, and density is restricted to 1-3 particles per mitochondrion, No background labelling is detected.

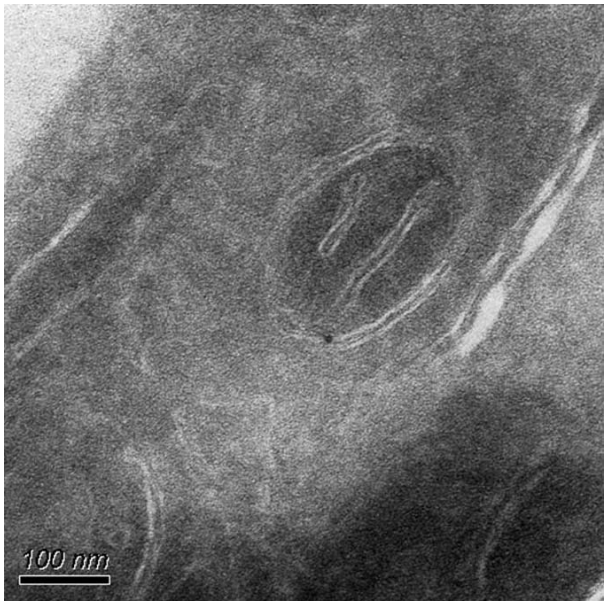

Figure 12. At higher magnification, localisation of anti-Kir6.2 labelling to mitochondrial inner membranes is evident.

For anti-Kir6.2-incubated cells, some specific immunogold labelling was apparent when the grids were compared with the controls. Small amounts of gold label were localized within the mitochondria (Figure 11, figure 12). In most cases this was limited to 1 – 3 gold particles per mitochondrion, and of the mitochondria that could be identified on the sections only a small proportion contained the label. However, very little background gold was evident, the amount being similar to that on the controls (Figure 9, figure 10), making it unlikely that the observed labelling represents non-specific binding of the primary antibody. Similarly, no gold was observed in mitochondria on the control sections, excluding the possibility that the observed mitochondrial labelling on the anti-Kir6.2-incubated grids represents non-specific binding of the secondary antibody.

The observations from the ultrastructural examination of ultrathin cryosections of SaOS-2 cells incubated with antibodies against Kir6.1 and Kir6.2 with the TEM therefore indicate that these cells do not contain

Kir6.1 but that small amounts of Kir6.2 are present and are localized to the mitochondria.

5. DISCUSSION

A variety of experimental methods have been used to study the existence and properties of mitoK_{ATP} in various cells and tissues.

Direct electrophysiological recordings of mitoK_{ATP} activity using patch clamp recording techniques is considered by many to offer the most convincing proof of the existence of these channels (1, 10, 42). However the method is technically challenging and is subject to uncertainty regarding the purity of the mitochondrial preparations (16).

Electrophysiological evaluations of purified mitoK_{ATP} reconstituted into bilayer lipid membranes (BLM), are similarly vulnerable to possible contamination of mitochondrial fractions with cell surface proteins (8, 9, 43-46).

Indirect assays of mitoK_{ATP} activity, such as measurements of mitochondrial volume, K^+ influx, flavoprotein oxidation or effects on respiration and $\Delta\Psi_m$ have also been used (2-4, 8, 12, 13, 47-49). However, many of these studies use one or more known pharmacological agonists or blockers, commonly diazoxide and 5-HD. The validity of this approach has been questioned due to the fact that most of these agents exert additional, mitoK_{ATP}-independent effects on mitochondrial and cellular function and much of this evidence therefore remains controversial (45, 46, 50-53).

Immunocytochemical methods, both at the light and electron microscopy level, offer the possibility of localizing channel subunits or other proteins to particular cellular compartments, and avoid the various problems associated with the use of isolated mitochondria and pharmacological mitoK_{ATP} modulators as described above. A number of workers have used specific antibodies to investigate the presence and subcellular location of putative mitoK_{ATP} subunits in various cells and tissues, and it is this approach that is employed in the present investigation to investigate the expression of Kir6.1 and Kir6.2 in the mitochondria of human osteoblast-like cells (6, 7, 9, 37-39, 53-61).

It is reasonable to assume that mitochondrial K^+ channels are rare, given the low conductance of the mitochondrial membrane towards potassium, and a highly sensitive immunolabelling protocol is therefore required to allow localization of channel subunits at subcellular level (62).

In this investigation the method of immunolabelling ultrathin thawed cryosections for transmission electron microscopy, commonly referred to as the Tokuyasu technique, was used (63). This technique has the advantage of allowing excellent penetration of antibodies into the section, giving enhanced labelling sensitivity over resin-embedding methods, whilst retaining good ultrastructural preservation (64, 65). This method has been shown to be extremely sensitive, and is particularly effective for immunolabelling of sparse membrane-bound antigens (66).

Immunogold labelling using a goat anti-Kir6.1 antibody, and a gold-conjugated anti-goat secondary failed to detect any specific Kir6.1 labelling in SaOS-2 cells by electron microscopy.

In rat cardiomyocytes, immunogold labelling with the same anti-Kir6.1 antibody produced low levels of specific mitochondrial label. This is broadly in agreement with other immunological studies using anti-Kir6.1 antisera in rat cardiac tissue although we did not observe the strong mitochondrial labelling reported by these workers (37-39). This difference in label density may be attributable to differences in experimental technique, or to differences in the antibodies used, although two of the studies used commercial antisera from the same manufacturer as those used in this investigation (Santa Cruz Biotechnology, Inc. California, USA) (38, 39). Alternatively, the dense labelling reported in these studies may reflect in part a tendency that we have observed for these antisera to produce excessive background labelling unless careful efforts are made to reduce this. We found it necessary to treat the Formvar coated grids by glow discharge prior to use, block specimens on a medium containing serum from the species in which the secondary antibody was raised and use the highest possible dilutions of primary and secondary antisera in order to reduce background staining to acceptable levels, given the low density of the specific staining.

Nonetheless, the anti-Kir6.1 antibody was shown to be capable of specifically labelling rat heart mitochondria using the experimental protocol described and, taking this as a positive control, the negative results obtained with the same antibody in SaOS-2 cells would appear to demonstrate that Kir6.1 is not present in these cells.

The anti-Kir6.2 antibody detected specific mitochondrial labelling of rat cardiomyocytes with the immunogold technique, in agreement with the findings of previous investigations and confirming that the antibody used was suitable for investigating Kir6.2 expression in SaOS-2 cells (37-39).

Immunogold electron microscopy of SaOS-2 cells revealed sparse mitochondrial expression of Kir6.2 in these cells, with negligible levels of background label.

Therefore, the results of the present investigation demonstrate that Kir6.2 is expressed in SaOS-2 cells, and that a very small amount of this subunit is present within the mitochondria. This finding is consistent with the hypothesis that osteoblast-like cells may contain mitoK_{ATP} channels, although these appear to be present in extremely low abundance under the conditions to which the cells used in this study had been exposed.

An improved understanding of the complex processes and pathways involved in osteoblast apoptosis may allow the identification of potential therapeutic targets to combat disorders of bone turnover such as osteoporosis, and if mitoK_{ATP} is found to play a significant role in this process, pharmacological modulation of this channel may represent a novel therapeutic approach to their management, similar to the increasing clinical role of mitoK_{ATP} openers in the protection of cardiac cells from apoptosis during ischemia-reperfusion injury.

6. CONCLUSIONS

1. Human osteoblast-like (SaOS-2) cells do not contain Kir6.1.

2. SaOS-2 cells contain Kir6.2, with small amounts present in the mitochondria under experimental conditions.

3. SaOS-2 cells may therefore contain mitoK_{ATP} channels in which Kir6.2 is the pore-forming subunit, although it appears that these channels are likely to be present in extremely low abundance at basal levels.

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