Activation and Inhibition of ATP-sensitive K⁺ Channels by Fluorescein Derivatives*

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Fluorescein derivatives are known to bind to nucleotide-binding sites on transport ATPases. In this study, they have been used as ligands to nucleotide-binding sites on ATP-sensitive K⁺ channels in insulinoma cells. Their effect on channel activity has been studied using stepwise nucleotide exchange and patch-clamp techniques.

Fluorescein derivatives have two opposite effects. First, like ATP, they can inhibit active ATP-sensitive K⁺ channels. Second, they are able to reactivate ATP-sensitive K⁺ channels subjected to inactivation or "run-down" in the absence of cytoplasmic ATP. Therefore reactivation of the inactivated ATP-sensitive K⁺ channel clearly does not require channel phosphorylation as is commonly believed.

The results indicate the existence of two binding sites for nucleotides, one activator site and one inhibitor site. Irreversible binding at either the inhibitor or the activator site on the channel was obtained with eosin-5-maleimide, resulting in irreversible inhibition or activation of the ATP-sensitive K⁺ channel respectively. The irreversibly activated channel could still be inhibited by 2 mM ATP.

After activation by fluorescein derivatives, ATP-sensitive K⁺ channels become resistant to the classical blocker of this channel, the sulfonamide glybenclamide. Negative allosteric interactions between fluorescein/nucleotide and sulfonylurea-binding sites were suggested by results obtained in [³H]glybenclamide-binding experiments.

Potassium channels that are sensitive to intracellular variations of ATP (K<sub>ATP</sub>) are important for the regulation of insulin secretion from pancreatic β-cells. In the absence of extracellular glucose, the β-cell is electrically silent. When glucose is raised to physiological concentrations (10 mM), the intracellular concentration of ATP increases, K<sub>ATP</sub> channels close, and the cell membrane depolarizes, giving rise to generation of action potentials and Ca<sup>2+</sup> influx (1–3). The ensuing Ca<sup>2+</sup>-stimulated insulin release by β-cells (4–7) ultimately leads to a reduction of blood glucose levels. K<sub>ATP</sub> channels are also present in heart (5, 6), skeletal muscle (7), and neuronal cells (8–10).

Intracellular ATP has a dual action on K<sub>ATP</sub> channels: (i) it closes the channel and (ii) it is necessary for the maintenance of channel activity. K<sub>ATP</sub> channel activity in β-cell and heart cell membranes decreases following patch-excision (run down) if low concentrations of Mg<sup>2+</sup>-ATP are not present in the intracellular solution. Channel activity is recovered after exposure of the patch to Mg-ATP followed by wash out of ATP (6, 11–16).

Inhibition of K<sub>ATP</sub> channels by ATP does not require Mg<sup>2+</sup>-ions, and nonhydrolyzable ATP analogs behave as ATP itself, indicating that ATP hydrolysis is not involved in channel inhibition (11, 13–23). The inhibitory action of ATP is reduced in the presence of ADP, suggesting that the ATP/ADP ratio may be more important than the intracellular concentration of ATP in regulating channel activity in the intact cell (11, 12, 17, 20, 21, 24).

Sulfonylureas are drugs that restore insulin secretion in patients affected by non-insulin-dependent diabetes mellitus. They have been shown to block the K<sub>ATP</sub> channel selectively (25–27). The sulfonylurea receptor is probably located on the K<sub>ATP</sub> channel, since the channel in excised patches is consistently blocked by sulfonylureas.

Fluorescein and its derivatives (FDs) have been used to label nucleotide-binding sites in various ATPases such as Ca<sup>2+</sup>-ATPases in the sarcoplasmic reticulum (28, 29) and erythrocyte (30), (Na<sup>+</sup>,K<sup>+</sup>)-ATPases (31–36), and (H<sup>+</sup>,K<sup>+</sup>)-ATPase (37). Eosin has been found to be a particularly useful tool to analyze the properties of ATP-binding sites on the (Na<sup>+</sup>,K<sup>+</sup>)-ATPase (32). This work shows that fluorescein derivatives induce both activation and inhibition of the K<sub>ATP</sub> channel in HIT-T15 insulinoma cells. Similar results were obtained with cardiac cells.

EXPERIMENTAL PROCEDURES

Materials—Phlophone B, oligomycin, ATP, ATP-γ-S, penicillin, and streptomycin were from Sigma. Spirit soluble eosin was from Aldrich. Eosin was from BDH Chemicals, Ltd. Fluorescein, 2',7'-dichlorofluorescein and Bengal rose B were from Fluka Chemical Corp. Eosin-S-maleimide was from Molecular Probes, Inc. Ham's F-12k medium was from GibCO. Gluiberclamide was from Hoechst. *RbCl was from Amersham Corp. All other reagents were of the purest grade available. All fluorescein derivatives are prepared fresh every day and utilized within 2–3 h, due to their photolability. Usually, 100 mM solutions were prepared in 100% dimethyl sulfoxide and then diluted with buffer to the given concentrations.

Cell Culture—HIT-T15 β-cells (ATCC, passage number 59) were cultured in Ham's F-12k medium supplemented with 10% dialyzed horse serum, 2.5% fetal calf serum (both inactivated), 100 units/ml penicillin, 0.1 mg/ml streptomycin, at 37 °C in humidified 5% CO<sub>2</sub>, 95% air. The culture medium was changed every 3–4 days. Culture flasks (Corning, 225-cm<sup>2</sup>) were used with 14–16 × 10<sup>6</sup> cells in 100 ml of medium. Cells were harvested weekly using trypsin-EDTA. Cells were seeded in 24-well plates (Falcon 3017) at a density of 3–5 × 10<sup>3</sup>
cells/well/ml 2-4 days before 45Rb+ efflux experiments, and the culture medium was supplemented with 7.4 kBa = 0.2 μCi/ml. 24-well plates were treated with 50 μg/ml polymyxin and washed three times before use with a medium containing 116 mM NaCl, 5.3 mM KCl, 5 mM d-glucose, 8 mM NaH2PO4, 22.6 mM NaHCO3, and 10 mg/ml phenol red. Cells were used from passage numbers 62-73. Rat cardiac ventricular cells were dispersed in a Ca2+-free medium containing 0.01% collagenase, followed by gentle mechanical agitation. Details of the method have been described elsewhere (38).

45Rb+ Efflux Experiments—After removing the culture medium, cells were preincubated in a medium containing 130 mM NaCl, 10 mM KCl, 1.5 mM CaCl2, 0.8 mM MgCl2, 20 mM Hepes/NaOH, pH 7.5, supplemented with 3.7 kBa = 0.1 μCi/ml. After 120-150 min of incubation, efflux experiments were started by removing the medium containing 45RbCl and replacing it with the same medium lacking 45RbCl. Drugs were preincubated in the plate wells in the presence of 45RbCl. Efflux was stopped at the indicated times by washing the cells with 1 ml of the 45RbCl-free medium. Cells were then extracted with 1 ml of 0.1 M NaOH and counted for radioactivity. Experiments were carried out at 37°C.

Gilbenclamide Binding—Binding experiments were carried out according to Schmid-Antomarchi et al. (25). Membranes (0.5-0.6 mg/ml final concentration) were incubated in 50 mM Hepes/NaOH, pH 7.3, with 1 nM [3H]Gilbenclamide (1.85 TBq = 50 Ci/mmol; Hoechst-Roussel) in the presence of increasing amounts of Bengale rose or phloxine B for 1 h on ice. 0.5-ml samples were vacuum-filtered through Whatman GF/C filters and washed with 100 mM Tris/HCl, pH 7.5, at 4°C. Filters were incubated with 5 ml of Biofluor (Du Pont-New England Nuclear) and counted for radioactivity.

Electrophysiology—Current-clamp experiments were carried out on HIT-T15 β-cells and heart cells using the whole cell suction pipette technique (39). Single-channel currents were recorded from inside-out and outside-out membrane patches, and their membrane potentials were clamped at 0 mV using a voltage-clamp amplifier (Bio-Logic, France). In all experiments, the intracellular solution contained 150 mM KCl, 1 mM MgCl2, 2 mM EGTA, 10 mM Hepes-KOH, pH 7.2. The extracellular solution was 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM Hepes/NaOH, pH 7.3. Pipettes were coated with Sylgard resin to reduce current noise. Electrical signals were digitized by a digital oscilloscope (Nicolet Instrument Corp.) and stored on hard disc by computer (Hewlett-Packard Co.) for further analysis. Experiments were carried out at room temperature (~25°C).

RESULTS

The structures of FDs that have been used in this study are presented in Fig. 1.

One of the FDs, eosin, has been shown to inhibit the (Na+,K+)-ATPase by occupying the nucleotide-binding site of the enzyme (31, 32). Its action on KATP channel activity in outside-out membrane patches excised from HIT-T15 insulinoma cells is presented in Fig. 2A (n = 6). The patch, excised with a pipette containing a K+-rich and ATP-free medium, contained two active KATP channels that were completely and reversibly inhibited by application of 2 mM eosin to the extracellular face of the membrane. Subsequent application of the now classical KATP channel blocker glibenclamide also inhibited channel activity.

Under the experimental conditions of Fig. 2A, it was the absence of ATP in the cytoplasmic medium that induced KATP channel activity. KATP channels can also be activated in the presence of ATP in the cytoplasmic medium, provided that both ADP and Mg2+ are present to relieve ATP inhibition (11). These conditions were used for the results shown in Fig. 2B-D, where channels were opened by supplying 0.5 mM ATP, 2 mM ADP, and 2 mM Mg2+ to the intracellular medium. Membrane potentials were recorded using the whole cell suction pipette technique (39). Activation of KATP channels hyperpolarized HIT-T15 cells to -50 ± 5 mV. Extracellular application of 100 μM of the FD phloxine B to these hyperpolarized cells provoked depolarization, generation of action potential, and KATP channel activity.

FIG. 1. Molecular structure of fluorescein derivatives used in this study. Compound 1, fluorescein; compound 2, ethylfluorescein; compound 3, 2',7'-dichlorofluorescein; compound 4, eosin (2',4',5',7'-tetrabromofluorescein); compound 5, phloxine B (2',4',5',7'-tetrabromo-3,4,5,6-tetrachlorofluorescein); compound 6, Bengale rose (2',4',5',7'-tetrachloro-3,4,5,6-tetrachlorofluorescein).

FIG. 2. Fluorescein derivatives inhibit KATP channels in HIT-T15 insulinoma cells. A, outside-out membrane patches were excised from HIT-T15 cells, clamped at 0 mV, and KATP channel activity was recorded. The pipette contained a K+-rich, ATP-free solution, and the bath contained a Na+-rich solution. Application of 2 mM eosin at the extracellular face of the membrane slowly reduced channel activity. Upon washing of eosin, channel activity reappeared that had been inhibited by subsequent extracellular application of 100 nM of the KATP channel blocker glibenclamide. Membrane potentials of HIT-T15 insulinoma cells were recorded using the suction pipette technique (B and D). The insulinoma cells were intracellularly dialyzed with a solution containing 2 mM ADP, 0.5 mM ATP, and 2.5 μg/ml oligomycin. After passing to the whole cell configuration, cells hyperpolarized and their membrane resistances decreased, due to opening of the KATP channels (B and D). Upon extracellular application of 100 μM phloxine B, cells depolarized and membrane resistance increased. Extracellular application of 2 mM ADP was without effect (D). Downward deflections in B and D were induced by injection of 300-ns, 5-pA current pulses, KATP channel activity in outside-out membrane patches, excised with a pipette containing 2 mM ADP and 0.5 mM ATP, was rapidly inhibited by 100 μM phloxine B (C). Channel activity that reappeared upon washing was blocked by 20 μM glibenclamide. Data shown in the upper trace of C were sampled at a slow rate (50 ms/sample). Sections of the same recording are shown underneath at an expanded time scale (5 ms/sample). Upward deflections in A and C correspond to channel openings.
potentials, and an increase of membrane resistance (Fig. 2B, n = 5). Outside-out-membrane patches were excised from HIT-T15 cells using the same pipette solution, containing ATP, ADP and Mg²⁺, as for whole cell recordings (Fig. 2D) to show more directly that the phloxine B-induced depolarization was due to KₐtP channel inhibition. The inhibition by 100 µM phloxine B (Fig. 2C, n = 3) was partially reversible, and channel activity, which reappeared after wash out, could be blocked by a high concentration (20 µM) of glibenclamide. During or shortly after exposure to phloxine B, 100 nM glibenclamide was without effect on the activity of KₐtP channels (n = 8), suggesting that the sensitivity of the KₐtP channel to sulfonyleureas was altered by phloxin treatment.

Since in the whole cell experiments presented in Fig. 2 phloxine B was applied extracellularly, we had to consider the possibility that the FDs could exert their inhibitory action via a putative adenyl nucleotide (ADP)-binding site, which has been proposed to be present on the extracellular domain of the KₐtP channel (40). Fig. 2D shows that under the same conditions as in Fig. 2B, 2 mM extracellular ADP had neither an effect on membrane polarization nor did it prevent phloxine B-induced depolarization. Therefore, it seems likely that the FDs, phloxine B and eosin, permeate the membrane and occupy regulatory nucleotide-binding sites situated on the cytoplasmic face of the KₐtP channel. Hydrophobic compounds such as fluorescein and its derivatives easily cross the cellular membrane.

The ⁶⁸Rb⁺ efflux technique has been previously used with success for the analysis of KₐtP channel activity in insulinoma cells (25). ⁶⁸Rb⁺ efflux induced by ATP depletion is fully blocked by glibenclamide (25). In experiments described in Fig. 3, KₐtP channels were opened by intracellular ATP depletion using oligomycin to inhibit the mitochondrial ATP synthetase as previously described (25). Increasing concentrations of phloxine B inhibited the oligomycin-induced ⁶⁸Rb⁺ efflux (Fig. 3). The Kₐ₅₀ value for this inhibitory effect was 6 ± 2 µM. Other FDs were also found to inhibit KₐtP channels with the following Kₐ₅₀ values: 63 ± 20 µM for fluorescein, 158 ± 50 µM for ethyleosin and dichlorofluorescein (not shown), 32 ± 5 µM for eosin, and 4 ± 1 µM for Bengal rose (not shown).

Electrophysiological and ⁶⁸Rb⁺ efflux experiments described until this point have shown that FDs could inhibit KₐtP channel activity under two conditions: (i) when the channel was made active by removing intracellular ATP and (ii) when the channel was maintained active in the presence of ATP, ADP, and Mg²⁺.

One of the important properties of the KₐtP channel in various tissues is the gradual inactivation that follows patch excision in an ATP- (or ADP-) free medium, a process known as channel "run down." It has been shown that KₐtP channel activity in membrane patches subjected to run down in the absence of nucleotides is restored following a transient exposure (1–2 min) of the intracellular face of patches to Mg-ATP (13). As was already mentioned in the Introduction, this strongly suggests that Mg-ATP plays a role in the maintenance of an activatable conformation of the KₐtP channel. Since Mg-ATP can "refresh" a run down channel, it was expected that the FDs, known to be ligands of Mg-ATP-binding sites in membrane ATPases, could mimic the refreshment produced by Mg-ATP.

Fig. 4A shows recordings from an inside-out patch containing a single KₐtP channel. In this experiment, soon after patch excision in a K⁺-rich and ATP-free medium, channel activity declined until the patch became silent after 60 s. The KₐtP channel was then in the inactivated (run down) state, which is indicated as the Cᵢ state (Fig. 4A). In this particular experiment, which was one out of a series of 22 experiments with similar results, the first application of 100 µM phloxine B had no effect on the inactivated channel. A similar lack of effect would have been observed if the inactivated channel had been exposed to an inhibitory concentration of ATP. The channel was then in the Cᵢ state, where the FD occupies the inhibitory site(s). However, if phloxine B by itself did not reanimate the KₐtP channel, wash out of phloxine B transiently restored channel activity. Again, the same behavior would have been seen after exposure to 2 mM ATP in the presence of Mg²⁺ followed by wash out. Hence, after phloxine B wash out, the channel was converted from the Cᵢ state into the active Cᵢ state, where the FD was assumed to occupy an activating site, and then slowly returned to the inactivated Cᵢ form. A second application of phloxine B then directly activated the channel for a period of about 2 min before the channel activity decreased again. Reactivation of the channel by phloxine B in the latter case suggests that during this second phloxine B application, the FD first occupied the activating FD site on the channel and converted it in the Cᵢ state before occupying an inhibitory site a little while later. A simple interpretation of these results at this stage is that two FD-binding sites are present on the KₐtP channel. Occupation of one of them activates the channel (Cᵢ → Cᵢ transition). On longer exposures, occupation of an inhibitory site by a second FD molecule closes the channel (Cᵢ → Cᵢ transition). Wash out of phloxine B transiently restores channel activity because of the conversion of the Cᵢ state.
This experiment, which was one out of a series of channel inactivation (conversion to the phloxine form) in HIT-T15 cells in a K+-rich medium by a pipette containing a Na+-rich solution and voltage-clamped at 0 mV. A, soon after patch excision, activity in a patch containing a single K\textsubscript{ATP} channel disappeared. During the first application of 100 μM phloxine B, the patch remained silent, but channel activity reappeared upon washing at the FD. A second application of phloxine then activated the K\textsubscript{ATP} channel. Note that the open channel probability (P) during the second application drops to a low level, to increase upon wash out. The sketch and symbols above the figure summarize the interpretation of the results as given in the text. In short: C\textsubscript{i}, inactivated or run down channel; C\textsubscript{FDa}, inactivated channel having inhibitory and excitatory nucleotide-binding sites occupied by FD; C\textsubscript{FDa}, activated channel having only its activatory nucleotide-binding site occupied by FD; C\textsubscript{i}, phloxine B-activated K\textsubscript{ATP} channels could be reversibly inhibited by an additional application of 2 mM ATP to the cytoplasmic side of the membrane patch. 100% on the ordinates (P) corresponds to a single channel that remains open during a 20-s sample period. Downward deflections in the single-channel traces correspond to channel opening. The numbers in the P(t) graph identify the time at which the records, shown at the right-hand side, were taken. The open and closed states of the channel are indicated by O and C, respectively.

Fig. 4B focuses on the activatory action of phloxine B. In this experiment, which was one out of a series of 12 experiments with similar results, the inside-out membrane patch contained at least three K\textsubscript{ATP} channels. The very low activity observed just after patch excision (trace 1) was due to partial channel inactivation in the ATP-free medium. Application of phloxine B increased K\textsubscript{ATP} channel activity (C\textsubscript{FDa} form). Washing first produced a further slight increase of channel activity and then led to a gradual loss of activity as a result of channel inactivation (conversion to the C\textsubscript{i} form). Fig. 4C shows that phloxine B-induced channel activation could be inhibited by 2 mM ATP. The open channel probability (P open) of control channels measured early after patch excision was estimated to be 0.34 ± 0.08. P-open reduced to 0.00 with time (run down). Maximum P-open following phloxine B application averaged 0.41 ± 0.32 and reduced to P-open = 0.09 ± 0.11 during sustained applications. Upon removal of phloxine, channel activity transiently obtained an average P-open value of 0.59 ± 0.23. Hence both “on” and “off” responses to phloxine are transient.

Fig. 5 provides a direct demonstration of the presence of both activating and inhibitory nucleotide/FD binding sites on the intracellular face of the K\textsubscript{ATP} channel. These data were obtained with the FD, eosin-maleimide (EMA). This compound has been used as an affinity label reagent to covalently form into the C\textsubscript{FDa} form, which on further washing, leads to the C\textsubscript{i} form.

**Fig. 4.** The fluorescein derivative phloxine B transiently activates K\textsubscript{ATP} channels. Inside-out membrane patches were excised from HIT-T15 cells in a K\textsuperscript{+}-rich medium by a pipette containing a Na\textsuperscript{+}-rich solution and voltage-clamped at 0 mV. A, soon after patch excision, activity in a patch containing a single K\textsubscript{ATP} channel disappeared. During the first application of 100 μM phloxine B, the patch remained silent, but channel activity reappeared upon washing at the FD. A second application of phloxine then activated the K\textsubscript{ATP} channel. Note that the open channel probability (P) during the second application drops to a low level, to increase upon wash out. The sketch and symbols above the figure summarize the interpretation of the results as given in the text. In short: C\textsubscript{i}, inactivated or run down channel; C\textsubscript{FDa}, inactivated channel having inhibitory and excitatory nucleotide-binding sites occupied by FD; C\textsubscript{FDa}, activated channel having only its activatory nucleotide-binding site occupied by FD; C\textsubscript{i}, phloxine B-activated K\textsubscript{ATP} channels could be reversibly inhibited by an additional application of 2 mM ATP to the cytoplasmic side of the membrane patch. 100% on the ordinates (P) corresponds to a single channel that remains open during a 20-s sample period. Downward deflections in the single-channel traces correspond to channel opening. The numbers in the P(t) graph identify the time at which the records, shown at the right-hand side, were taken. The open and closed states of the channel are indicated by O and C, respectively.

**Fig. 5.** Eosin-maleimide binds covalently to K\textsubscript{ATP} channels, either inhibiting or activating them irreversibly. Inside-out membrane patches were treated with 25 μM EMA + 5 μM ATP for two min, and channel activity was recorded at 0 mV. A, channel activity in control patches is completely suppressed by exposure of the intracellular face of the membrane to 2 mM ATP. Upon wash out, ATP, channel activity reappeared (upper traces). In nine out of 23 inside-out patches, exposure of the intracellular face of the membrane resulted in irreversible K\textsubscript{ATP} channel inhibition, since 2 mM of ATP was unable to restore channel activity (lower traces). B and C, in seven out of 23 patches, irreversible K\textsubscript{ATP} channel activation by EMA was observed. Current density histograms from a single inside-out membrane patch (C) show that after EMA treatment (2) followed by a washing (3), channel activity was considerably higher (3) than in the control period (1). The channel was fully (4) and reversibly (5) blocked by 2 mM of ATP, but had become less sensitive to glibenclamide (100 nM) (6). The absence in C are composed of 100 bins of 0.2 pA width. The ordinates, representing probability per bin, are scaled as indicated. The peaks around 0 pA represent the closed channel probabilities, other peaks indicate one, two, or more channels are open. The open and closed states of the channel are indicated by O and C, respectively.
Experimental points inhibited by increasing amounts of Bengale rose. The value was determined in the presence of 100 nM glibenclamide. Binding of 1 nM [3H]glibenclamide to HIT-T15 cell membranes has also been observed recently, with a \( K_{\text{d}} \) value for ADP of about 1.5 mM (40).

Beside pancreatic \( \beta \)-cells, \( K_{\text{ATP}} \) channels are present in a variety of other tissues, such as heart, skeletal muscle, smooth muscle, and brain (1, 5–9, 41). The modulation of \( K_{\text{ATP}} \) channel activity by nucleotides has been particularly well studied both in \( \beta \)-cells and cardiac cells (6, 11–21, 23, 24, 27).

Although differences in properties of \( \beta \)-cell and cardiac \( K_{\text{ATP}} \) channels have been identified, it is well established that the general characteristics of the channel, such as its sensitivity to various types of nucleotides, its inactivation in ATP-free solution, and its sensitivity to sulfonylureas, are similar. It was therefore expected that FDs could also act on cardiac \( K_{\text{ATP}} \) channels.

Fig. 7 shows that 100 \( \mu \)M phloxine B reversibly inhibited \( K_{\text{ATP}} \) channel activity in inside-out membrane patches excised from dissociated rat cardiac cells (n = 5), as FDs in insulinoma cells (Fig. 2). The \( K_{\text{ATP}} \) channel activity reappeared on phloxine B washing and was rapidly inhibited by application of 2 mM ATP to the intracellular face of the patch.

![Fig. 6. Inhibition of specific [3H]glibenclamide binding to HIT-T15 cell membranes by Bengale rose and phloxine B. Binding of 1 nM [3H]glibenclamide to HIT-T15 cell membranes was inhibited by increasing amounts of Bengale rose (○) and by phloxine B (●). \( K_{\text{d}} \) values were 0.2 ± 0.1 and 2 ± 1 mM, respectively. The zero value was determined in the presence of 100 \( \mu \)M cold glibenclamide. Experimental points represent mean values of duplicates or triplicates.](image-url)

*Effects of Fluorescein Derivatives on \( K^+ \) Channels*
DISCUSSION

This work shows that FDs have modulatory effects on KATP channels in insulinoma and cardiac cells. They can both activate and inhibit KATP channels in insulinoma cells. Inhibition was observed under two sets of conditions: (i) when FDs were applied to active KATP channels soon after removal of cytoplasmic ATP and (ii) when FDs were applied to KATP channels which were maintained in the active form by an internal medium containing ATP, ADP, and Mg2+. Activation was observed when FDs were applied to (partially) inactivated KATP channels.

The results can be explained by a single model with two nucleotide/FD-binding sites on the intracellular face of the KATP channel, one activatory site (A-site) and one inhibitory site (I-site). In this model, occupation of the A-site activates the channel, whereas occupation of the I-site or occupation of both A- and I-sites inhibits the channel.

The existence of two types of binding sites for FDs was more directly demonstrated by experiments with EMA (Fig. 5). EMA can covalently and irreversibly occupy the A- and I-sites. The irreversibly activated channel, with only its A-site occupied by EMA, could still be completely inhibited by ATP, showing that covalent modification of the A-site does not prevent channel inhibition by ATP at the I-site.

How do these results obtained with FDs relate to previous observations concerning modulation of channel activity by ATP and ADP? The modulation of KATP channel activity by ATP and ADP has been particularly well studied in β-cell and cardiac cells (reviewed in Refs. 6, 42, and 43). It has been shown that ADP and ATP exert a dual action on KATP channel activity. On the one hand, millimolar concentrations of either ADP or ATP, in either the absence or presence of Mg2+, inhibit KATP channel activity. On the other hand, low concentrations of Mg-ADP or Mg-ATP can reactivate (partially) inactivated KATP channels. For example, in cardiac cells, 100–250 μM ADP in the presence of Mg2+ activate partially run down KATP channels, whereas higher concentrations (>250 μM) are inhibitory (13, 19). These observations also suggest the existence of activatory and inhibitory nucleotide-binding sites on the KATP channel. The A- and I-sites would accept different forms of the ADP molecule. Whereas free ADP seems to be the preferred species for binding to the I-site, Mg-ADP is required at the A-site. The same holds for ATP. Mg-ATP can refresh the KATP channel after run down and free ATP or nonhydrolyzable ATP analogs are ineffective in this respect, whereas free ATP and ATP analog inhibit the channel (13, 24).

It has been suggested that KATP channel reactivation by Mg-ATP involves channel phosphorylation (12, 24, 42–45). A role for protein phosphorylation in the reactivation was suggested (i) by the fact that nonhydrolyzable ATP analogs are ineffective and (ii) by the requirement of Mg2+, which is necessary for kinase activity. The observation made in this paper that FDs can restore run down channel activity clearly indicates that channel reactivation or “refreshment” does not require channel phosphorylation.

Very little is known about the interactions between nucleotide-binding sites on the one hand and binding sites for sulfonylureas on the other hand. Results presented in Figs. 5 and 6 indicate that if the A-site is occupied by FDs, the channel has become insensitive to the sulfonylurea glibenclamide, a potent blocker of KATP channels (25). There is then clearly a negative allosteric interaction between FD binding at the A-site and glibenclamide binding to the KATP channel. This inhibitory effect of FDs on sulfonylurea interaction with the KATP channel was demonstrated by [3H]glibenclamide binding data (Fig. 6). These results are consistent with a very recent observation that intracellular ADP interferes with the ability of glibenclamide to block cardiac KATP channels (46).

Effects of Fluorescein Derivatives on K+ Channels

Finally this study suggests a way to covalently label nucleotide-binding sites on the KATP channel. Irreversible activation by EMA involves recognition of the nucleotidic A-site by the eosin moiety of EMA and subsequent formation of a covalent link by the maleimide part. Maleimides preferentially interact with sulphydryl groups, and it may be that nucleotide-binding sites on the KATP channel have essential–SH groups. Sulphydryl groups are also known to be essential components of the interaction of ATP with its regulatory site(s) in transport ATPases such as the (Na+,K+)-ATPase (reviewed in Ref. 49) and they are readily modified by N-ethylmaleimide. It has been shown recently that N-methylmaleimide, if applied to the cytoplasmic face of membrane patches from skeletal muscle, abolishes KATP channel activity irreversibly and that this inhibition is prevented by ATP (50). Radiolabeled EMA might be a useful tool to identify peptide sequences in the channel structure that contain the regulatory nucleotide-binding sites. Affinity labeling studies with this compound could also indicate whether the same or different subunit components carry nucleotide-binding sites and antidiabetic sulfonylurea-binding sites.

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