J. Membrane Biol. 154, 183–195 (1996)

The Journal of
Membrane
Biology

Role and Source of ATP for Activation of Nonselective Cation Channels by AlF Complex in Guinea Pig Chromaffin Cells

M. Inoue,[1] K. Ogawa[2], N. Fujishiro[1], A. Yano[1], I. Imanaga[1]
Departments of Physiology[1] and Anatomy[2], School of Medicine, Fukuoka University, Fukuoka 814-01, Japan

Received: 17 April 1996/Revised: 26 July 1996

Abstract. Intracellular dialysis with the solution containing the G protein activator, AlF complex, induced an inward nonselective cation current (I_{NS}) at −55 mV in chromaffin cells. Amplitudes of I_{NS} induced by dialysis with ATP-free AlF solutions progressively diminished as cells were pretreated with cyanide, a mitochondrial inhibitor. After a 10-min pretreatment, generation of I_{NS} by the AlF complex depended on exogenous ATP delivered from pipette solution. The relationship between amplitudes of I_{NS} and concentrations of MgATP was well expressed by a rectangular hyperbola with an EC_{50} of 0.265 mM. This result suggests that the cyanide treatment almost depleted ATP near the plasma membrane. On the other hand, a similar cyanide treatment of adrenal medullary preparations did not induce a marked decrease in cellular ATP content. GTP, ITP, or UTP could not substitute for ATP in generation of I_{NS} by the AlF complex. Similarly, the substitution of ATP with non- or poorly hydrolyzable ATP analogues did not aid in generating I_{NS}. Bath application of the kinase inhibitor, H-7 (100 μM), suppressed AlF-induced I_{NS} in a manner depending on intracellular Mg^{2+}. We conclude that ATP is a prerequisite for generation of I_{NS} as a phosphoryl donor and that mitochondria is the main source of ATP.

Key words: ATP — Mitochondria — Glycolysis — Nonselective cation channel — Phosphorylation — G protein

Introduction

The muscarinic receptor, probably M4 subtype, in guinea-pig chromaffin cells is coupled with a nonselective cation (NS) channel through a pertussis toxin-sensitive GTP binding (G) protein (Inoue & Kuriyama, 1991; Inoue & Imanaga, 1995*b*). Our previous studies (Inoue & Imanaga, 1993, 1995*b*) suggested that protein kinase and Mg^{2+}-dependent phosphatase are involved in activation and deactivation of the channel, respectively. Extracellular application of isoquinolinesulfonamide derivatives, which are known to inhibit serine/threonine protein kinases (Hidaka et al., 1984), reversibly inhibited muscarine-induced nonselective cation currents (I_{NS}), whereas infusion of orthovanadate, a nonspecific phosphatase inhibitor (Shenolikar & Nairn, 1991), into cells enhanced amplitude of the muscarine-induced I_{NS} and retarded the time course of deactivation after washout of the agonist. Moreover, these effects of orthovanadate were mimicked by a decrease in intracellular concentrations of Mg^{2+}. All these observations are consistent with our thesis, but individual results can be otherwise explained. Thus, the inhibition of I_{NS} by isoquinolinesulfonamides could be due to a channel block, as was suggested for the calmodulin antagonist-induced inhibition of voltage-sensitive Ca^{2+} and K^+ channels in NG 108-15 cells (Caulfield et al., 1991) and of Ca^{2+}-dependent K^+ channels in smooth muscle cells (Kihira et al., 1990). The potentiating effect of orthovanadate could be attributed to inhibition of ion-conducting ATPases, such as the Na^+, K^+ ATPase (Macara, 1980). The primary objective of the present experiment was to examine whether or not ATP is essential for activation of NS channels and, if so, what is a source of the ATP. Ion-transporting activity in the plasma membrane, such as the Na^+, K^+ ATPase (Mercer & Dunham, 1981; Balaban & Bader, 1984) and ATP-sensitive K^+ channel (Weiss & Lamp, 1989), was shown to be regulated by ATP derived preferentially from glycolysis. We also investigated whether ATP is utilized as an energy source or a phosphoryl donor. This issue is recently gaining much attention (Ames & Lecar, 1992; Furukawa et al., 1994) since gatings of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^-

Correspondence to: M. Inoue

channels were proposed to be regulated by energy released by ATP hydrolysis (Anderson et al., 1991; Baukrowitz et al., 1994). Our present results indicate that mitochondrial ATP is involved in activation of NS channels under aerobic conditions and is used as a phosphoryl donor.

Materials and Methods

Current Recording

The procedures to obtain single chromaffin cells and record whole-cell currents were almost the same as described previously (Inoue & Imanaga, 1995*a*). Briefly, the adrenal medullae were obtained by removing the adrenal cortex and cut into several pieces. These preparations were treated with collagenase for 30 min and then cells were dissociated by pipetting. Whole-cell currents were recorded at −55 mV using the tight-seal patch clamp technique. After recordings with a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA), currents were passed through a high-cut filter of 5 or 3 Hz and then fed into a pen recorder. The resistance of a patch pipette was 1.5–2.0 MΩ, when filled with internal solution. The series resistance in whole-cell recordings was generally about two or three times larger than the pipette resistance.

Solutions

The standard external solution contained (in mM): 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.53 NaH_2PO_4, 5 D-glucose, 5 HEPES, and 4 NaOH. In CN solution, 5 mM NaCl was replaced with 5 mM NaCN and the glucose was removed, and in 2-deoxyglucose (DG)-containing solution (DG solution), 5 mM DG substituted for the glucose. The pH of all external solutions was adjusted to 7.4. The standard aluminum fluoride (AlF) complex-containing pipette solution (AlF solution) consisted of (in mM): 110 K aspartate, 10 KF, 10 KCl, 5 EGTA, 5 HEPES, 2 Na_2ATP, 0.1 $AlCl_3$, and 12 KOH. In other nucleotide-containing AlF solutions, it substituted equimolarly for ATP, and in an ATP-free solution, 2 mM ATP in the standard solution was replaced with 4 mM NaCl. The pH in all the pipette solutions was 7.2. Unless otherwise noted, the concentration of free Mg^{2+} ($[Mg^{2+}]$) in AlF solutions was kept at 12 μM by adding an appropriate amount of $MgCl_2$ (Sakai et al., 1992; Sillén et al., 1971; Perrin, 1979). In a nominally Mg^{2+}-free AlF solution, no $MgCl_2$ was added. The liquid junction potential between the external and internal solutions was about −12 mV and was corrected for membrane potential measurements. All the experiments were done at 23–25°C. The results are expressed as means ± SEM and Student's *t* test was used to determine the statistical significance ($P < 0.05$).

Electron Microscopy

Adrenal glands were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr at room temperature. Specimens were post-fixed in 4% OsO_4, dehydrated and embedded in Epon. This sections were doubly stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 electron microscope.

Measurements of Nucleotides

In each run of measurements, 4 ($n = 20$) or 6 ($n = 2$) guinea-pigs were killed by a blow to the neck and the adrenal glands were removed. The adrenal medulla was obtained by removing the adrenal cortex with fine forceps and microscissors under microscopic vision. A total of 8 or 12 adrenal medullae were weighed individually (each, 3 to 6 mg) and these were divided into two groups to have a roughly equal weight, then each medulla was cut into two or three pieces. The two groups of preparations were incubated first in the standard solution for 10 min, then each group was incubated in either the standard solution or ATP depletion solutions (CN solution or 1 mM iodoacetate-containing DG solution) for 12–15 min or 50 min. During the incubation, temperature was kept at 24–25°C, but all other procedures were under ice-cooled conditions.

Contents of nucleotides in adrenal medullary preparations were measured with high performance liquid chromatography (HPLC). The HPLC system (Waters, Milford) was comprised of 600E multisolvent system connected with Radial Pak 8P SAX column, 484 tunable absorbance detector, and M741 data module. The adrenal medullary preparation was homogenized in 0.6 ml of 0.42 M $HClO_4$ and then centrifuged for 10 min at 4°C. A 0.5 ml volume of the supernatant was neutralizing by adding 0.25 ml of 1N KOH, then centrifuged for 3 min. After filtering, 10 μl of the supernatant was injected onto the HPLC column. For the first 2 min after sample injection, the column was equilibrated with the buffer A (7 mM KH_2PO_4 and 7 mM KCl, pH 4.0) and then a linear solvent gradient with an increase of 6.25% per min in composition of buffer B (250 mM KH_2PO_4 and 500 mM KCl, pH, 5.0) was applied. The flow rate was maintained at 4 ml min^{-1} throughout the separation. Nucleotides were detected by UV spectroscopy at 254 nm and concentrations were calculated based on the chromatogram of standard solution consisting of adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP), each at 0.1 mM.

Chemicals

Sources of chemicals are indicated: adenylyl-imidodiphosphate tetralithium salt (AMP-PNP), adenosine-5′-*O*-(3-thiotriphosphate) tetralithium salt (ATPγS), and guanosine 5′-triphosphate dilithium salt (GTP) (Boehringer Mannheim, Germany); adenosine 5′-triphosphate disodium salt (ATP), uridine 5′-triphosphate sodium salt (UTP), and inosine 5′-triphosphate sodium salt (ITP), 2-deoxyglucose (2DG), ADP sodium salt, AMP sodium salt, and iodoacetic acid sodium salt (Sigma Chemical); 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) (Seikagaku, Japan); sodium pyruvate (Katayama Chemical, Japan).

Results

Effects of Changes of Pipette Solution

Our previous studies (Inoue & Imanaga, 1993, 1995*b*) suggested that a phosphorylation step may be present somewhere in the signal pathway from muscarinic receptor to NS channel. To obtain further evidence for this hypothesis, ATP in the pipette solution was replaced with the nonhydrolyzable analogue of ATP, AMP-PNP, where the β, γ bridge oxygen of the phosphates is replaced with an imido group. If our hypothesis is tenable,

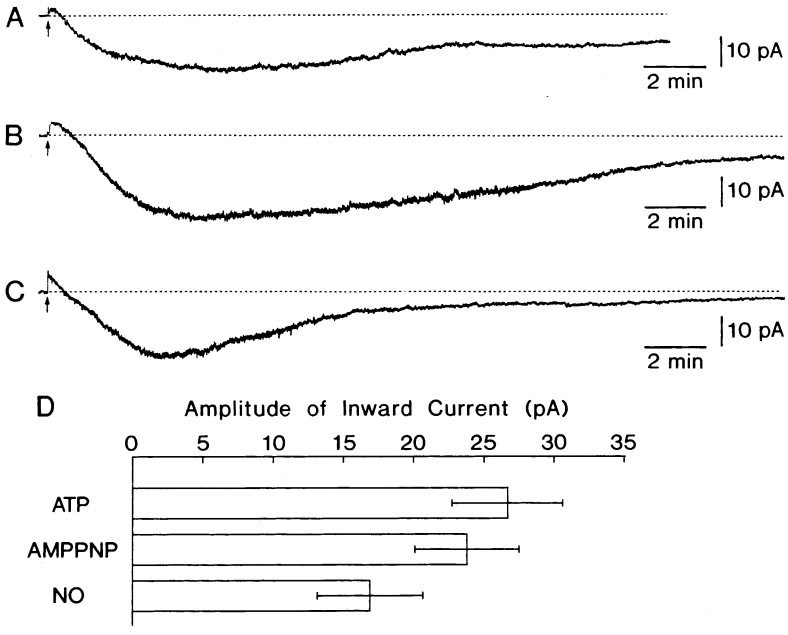

Fig. 1. Lack of effect on the AlF complex-induced nonselective cation current of removal of or replacement of ATP with AMP-PNP. (*A*), (*B*) and (*C*) whole-cell currents recorded with standard AlF solution containing 2 mM ATP, with AlF solution in which ATP was replaced equimolarly with AMP-PNP, and with AlF solution where ATP was replaced with 4 mM NaCl, respectively. $[Mg^{2+}]$ in all AlF solutions, 12 μM. In this and the following figures, holding potential was −55 mV, and arrows and broken lines represent the time of break-in and zero current level. (*D*) summary of maximum amplitudes of AlF-induced nonselective cation current (I_{NS}) in the presence of ATP ($n = 3$) or AMP-PNP ($n = 8$) and the absence of any nucleotide (NO; $n = 7$). Maximum amplitudes were measured with reference to the current level either at the latent period or 30 sec after break-in when the latency was not discernible.

the production of I_{NS} would diminish because of the low availability of substrates for protein kinase and the weak competitive inhibition by this analogue (Flockhart et al., 1984). I_{NS} was produced by intracellular application of the AlF complex (Inoue & Imanaga, 1995*a;* Inoue, Fujishiro & Imanaga, 1995), a G protein activator, since this method consistently induced large amplitudes of I_{NS} whereas under similar conditions, muscarinic receptor stimulation variably generated the current. Figure 1*A* shows a control whole-cell current recorded at −55 mV with a 2 mM ATP-containing AlF solution in which $[Mg^{2+}]$ was set to be 12 μM by adding 0.44 mM $MgCl_2$. The inward I_{NS} began to develop 15 ± 2 sec ($n = 3$) after intracellular access, reached a maximum at 310 ± 19 sec ($n = 3$) and decreased with a half decay time ($T_{1/2}$) of 273 ± 23 sec ($n = 3$), a time which is required for the half development of decline. The maximum amplitude of I_{NS} was 26.7 ± 4.0 pA ($n = 3$), measured with reference to the current level at the latent period or at 30 sec in the case where latency was not discernible (probably because of rapidly declining Ca^{2+}-dependent currents due to diffusion of EGTA; e.g., Fig. 1*C*). These values did not differ from those obtained with 5 mM ATP-containing AlF solutions with 12 μM Mg^{2+} ions (Inoue et al., 1995). When 2 mM ATP was replaced equimolarly with AMP-PNP, I_{NS} developed and decreased in a similar time course (latency, 26 ± 4 sec, $n = 6$; time of maximum, 341 ± 57 sec, $n = 8$; $T_{1/2}$, 380 ± 63 sec, $n = 7$). The maximum amplitude of I_{NS} (23.8 ± 3.7 pA, $n = 8$) was also not altered significantly. These results raise the possibility that the AlF complex-induced I_{NS} requires the binding of ATP or its analogue to a regulatory site of NS channel without hydrolysis, as is the case with ATP-sensitive K^+ channels (Spruce, Standen & Stanfield, 1987; Ohno-Shosaku, Zünkler & Trube, 1987). To examine this possibility, ATP in the standard AlF solution was replaced with 4 mM NaCl and the concentration of $MgCl_2$ was decreased from 0.44 mM to 0.015 mM. Figure 1*C* depicts a whole-cell current recorded under such conditions. The time course of I_{NS} development (latency, 19 ± 4 sec, $n = 5$; time of maximum, 272 ± 47 sec, $n = 7$) and the maximum amplitude of I_{NS} (16.9 ± 3.8 pA, $n = 7$) did not differ from observations under the other two conditions. The only difference was that the current elicited diminished significantly faster with a $T_{1/2}$ of 196 ± 20 sec ($n = 5$).

Various possibilities could account for the results shown in Fig. 1. First, the diffusional exchange between the cytoplasm and the pipette solution was not completed for the initial 5 or 6 min of whole-cell recordings. But this notion can probably be ruled out since infusion of the AlF complex consistently generated I_{NS} with short latencies of a few tens seconds. $AlF_3(OH)^-$ ions, the main effective form of the complexes (Antonny & Chabre, 1992), have a molecular weight of 101, being about one-fifth of that of ATP, and this difference may not account for the incomplete diffusional exchange, based on the diffusion times calculated with an empirical equation (Pusch & Neher, 1988; time constants for diffusion of AlF complex and ATP into cells under the present conditions, 14–18 sec and 25–32 sec). In addition, latencies observed in AlF-induced I_{NS} approximated the estimated time for the complex to diffuse into cells. To further confirm that a diffusional exchange readily occurs, $[Mg^{2+}]$ in AlF solutions were increased since an increase in $[Mg^{2+}]$ is expected to diminish the amplitude of I_{NS} and facilitate the time course of falling phase of I_{NS}, probably by enhancing Mg^{2+}-dependent phosphatase

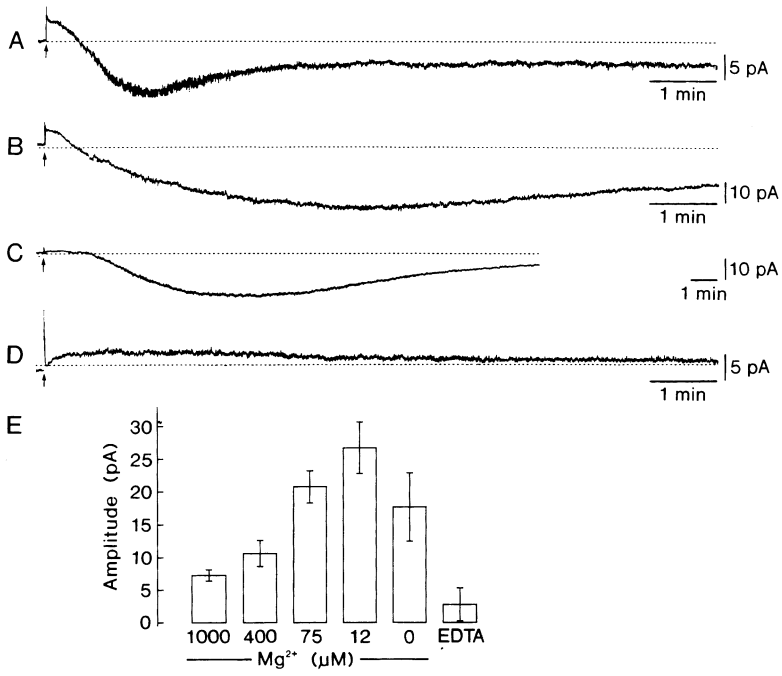

Fig. 2. AlF complex-induced I_{NS} in the presence of different concentrations of Mg^{2+}. (*A*) and (*B*) whole-cell currents recorded using standard AlF solutions with 1,000 μM Mg^{2+} and with 12 μM Mg^{2+}, respectively. (*C*) and (*D*) whole-cell currents from ATP-depleted cells recorded using standard AlF solutions with no $MgCl_2$ and with no $MgCl_2$ and 5 mM EDTA, respectively. Endogenous ATP in the cells was depleted with cyanide treatment for 10–12 min before and after break-in. (*E*) summary of maximum amplitudes of inward shift of current during dialysis with AlF solutions containing various concentrations of Mg^{2+} (n = 3–7 at each concentration). Data with 0 Mg^{2+} and EDTA were obtained from cells pretreated with CN solution. Maximum amplitudes of current or current levels 3 min after break-in were measured in the same manner as for Fig. 1.

activity (Inoue et al, 1995). Figure 2*A* and *B* show whole-cell currents recorded in the presence of 1,000 and 12 μM Mg^{2+} ions, respectively. In the former, an inward I_{NS} began to develop 12 sec after break-in, reached a maximum of 13 pA at 86 sec and then decayed rapidly, whereas in the latter, the current developed with the same latency but reached a maximum of 32.5 pA at 260 sec, then diminished much more slowly. Figure 2E summarizes maximum amplitudes of I_{NS} elicited at four different concentrations of Mg^{2+} ions from 1,000 to 12 μM; the I_{NS} increased with decreasing [Mg^{2+}].

Effects of Inhibition of ATP Production

The foregoing results indicate that the equilibration of ATP between the vicinity of NS channels and pipette solutions was hindered. One possible mechanism would be compartmentalization of ATP in or near the plasma membrane, as was suggested in red cells (Mercer & Dunham, 1981). Alternatively, ATP is continuously produced near the membrane by glycolysis, oxidative phosphorylation in mitochondria, or both so that the concentration of ATP does not readily equilibrate with that in pipette solutions. In either case, suppression of cellular production of ATP would result in disappearance of the current response to the AlF complex under conditions where ATP is not supplied exogenously. To test this inference, cellular ATP was depleted by glycolysis inhibitors or by an electron transport inhibitor before and during whole-cell recordings with ATP-free AlF solutions. When cells were exposed to glucose-free perfusate for up to 10 min, infusion of the AlF complex elicited an inward I_{NS} in a manner similar to that seen without the pretreatment (n = 4). However, when 5 mM cyanide, which binds tightly with cytochrome a in mitochondria, was added to the glucose-free solution, the generation of I_{NS} was diminished progressively with increases in pretreatment time (Fig. 3*A* and *B*). This cyanide effect was noted even with a 1-min pretreatment and appeared to develop maximally with that of 10 min (O in Fig. 3*E*). Another conspicuous effect of the cyanide pretreatment, which was observed in some, but not all cells, was generation of an outward current (e.g., Fig. 8*A*), probably due to an increase in intracellular [Ca^{2+}] (Wang, Randall & Thayter, 1994). The abolition of I_{NS} generation was also observed when glycolysis was inhibited by the application of 5 mM DG and 1 mM iodoacetate (Fig. 3*C* and □ in Fig. 3*E*), but not by that of DG alone (Fig. 3*D* and △ in Fig. 3*E*). In contrast to the cyanide pretreatment, this abolition of current generation was irreversible because of the irreversible binding of iodoacetate to a glycolytic enzyme. The observation that the suppression of ATP production by intervening at the two different sites, glycolysis and electron transport, both produced an inhibition of I_{NS} indicates that the failure of generation of I_{NS} can be attributed to depletion of endogenous ATP. Thus, to deplete endogenous ATP, cells were exposed to CN solution for 10 to 12 min.

The results of ATP depletion suggest that contents of ATP were markedly decreased by treatments with cyanide or DG and iodoacetate. Thus, we investigated how much the present procedures decreased contents of ATP in adrenal medullary preparations. Preparations in each run were divided into two groups and were incu-

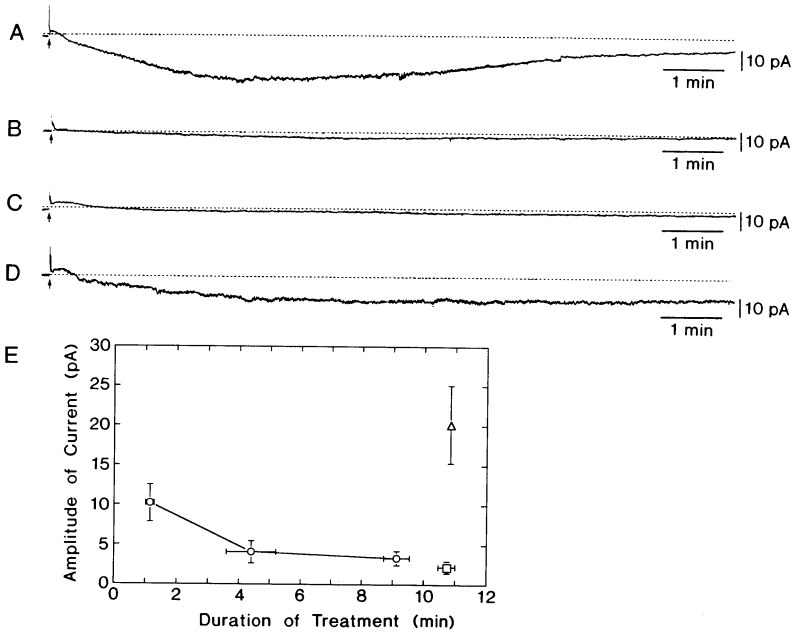

Fig. 3. Depletion of cellular ATP abolished generation of I_{NS} induced by the AlF complex. Whole-cell currents recorded from the cells pretreated with CN solution for 1 min (*A*) and 9 min (*B*) and with DG solution containing 1 mM (*C*) and 0 mM (*D*) iodoacetate for 10–12 min. All AlF solutions contained 4 mM NaCl instead of 2 mM ATP and 12 μM Mg^{2+} ions. CN and DG solutions contained 5 mM CN^- ions and 5 mM deoxyglucose, respectively (*see* Materials and Methods). (*E*) maximum amplitudes of I_{NS} induced by AlF complex in the absence of exogenous ATP in the cells pretreated with CN solution (o; $n = 4$–10), DG solutions containing 1 mM (□; $n = 7$) and 0 mM (△; $n = 4$) iodoacetate are plotted against the duration of treatment.

bated in standard solution for 10 min, then in either ATP depletion or standard solution for 12–15 min or 50 min. Subsequently, contents of ATP were measured using HPLC. The amount of ATP in preparations treated with depletion solutions was expressed as a fraction of that in control and was plotted against duration of treatments. Figure 4 shows that the decrease of ATP contents was unexpectedly small: in the case of cyanide, ATP contents were diminished by about 6% with a 12–15 min treatment and by about 13% with 50 min. Similarly, treatment with DG and iodoacetate resulted in a small diminution of ATP contents.

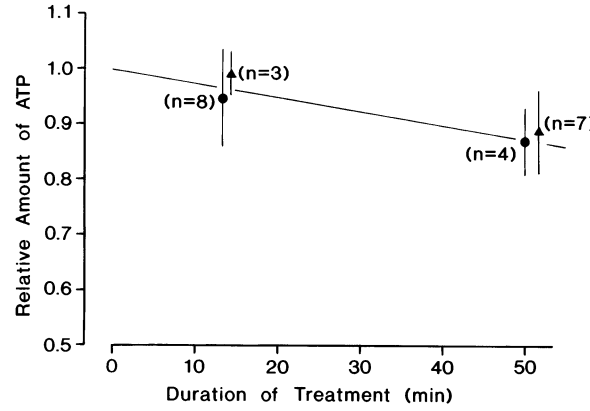

Fig. 4. ATP contents in adrenal medullary preparations incubated in ATP depletion solutions. Adrenal medullary preparations were incubated in standard solution for 10 min, then in either ATP depletion or standard solutions for 12–15 min or 50 min. Contents of ATP were measured with HPLC. The amount of ATP in test preparations is expressed as a fraction of that in control ones and is plotted against duration of treatment. ATP depletion solutions are CN solution (●) and 1 mM iodoacetate-containing DG solution (▲) (*see* Materials and Methods). Numbers in parenthesis represent number of observations.

ATP Concentration Dependence of I_{NS}

To examine the dependence on ATP concentrations, endogenous ATP was depleted by pretreatment with CN solution and whole-cell currents were recorded using AlF-solutions which contained various concentrations of ATP and 12 μM Mg^{2+} ions. The upper panel of Fig. 5 shows whole-cell currents in the presence of 2 mM (*A*), 600 μM (*B*), 200 μM (*C*), and 20 μM ATP (*D*). As the concentration of ATP added to AlF solutions decreased, the development of I_{NS} became small. These data are summarized in the lower panel where the maximum amplitude of I_{NS} was plotted against concentrations of added ATP (*E*) and MgATP (*F*). Data show a good fit with rectangular hyperbolas with a concentration causing half the maximum response (EC_{50}) of 850 μM and maximum current (I_{max}) of 24.5 pA (*E*) and with an EC_{50} of 265 μM and I_{max} of 24.7 pA (*F*). The EC_{50} for MgATP is much larger than K_m values of serine/threonine protein kinases with a broad spectrum for substrates (nucleotide-dependent kinases, 3.1-7.1 μM: Flockhart et al., 1984; protein kinase C, 4.4 μM: Wise et al., 1982). In contrast, it is compatible with the EC_{50} for ATP of CFTR Cl^- channels (270 μM: Anderson et al., 1991), and it approximates the concentration causing half the maximum inhibition (IC_{50}) for ATP in inhibiting ATP-sensitive K^+ channels in cardiac myocytes (100 μM; Noma, 1983) and skeletal muscle (135 μM: Spruce et al., 1987). All ATP-dependent enzymes may use MgATP (Eckstein, 1985), whereas in ATP-sensitive K^+ channels, the binding of ATP to a site of the channel results in inhibition (Spruce

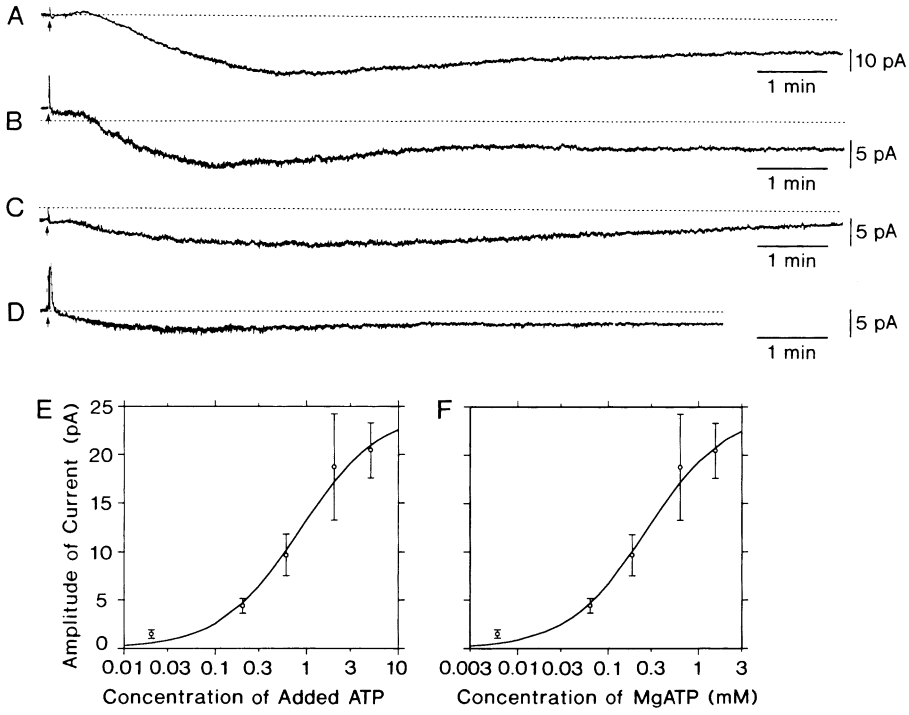

Fig. 5. Dependence of the AlF complex-induced I_{NS} on concentration of exogenous ATP in ATP-depleted cells. Whole-cell currents recorded with AlF solutions containing 2 (*A*), 0.6 (*B*), 0.2 (*C*), and 0.02 mM (*D*) ATP. [Mg^{2+}] in all AlF solutions, 12 μM. Endogenous ATP in this and the following figures was depleted with cyanide, as explained in Fig. 2. (*E*) and (*F*), maximum amplitudes of AlF-induced I_{NS} are plotted against concentrations of added ATP and MgATP, respectively. Data points (n = 4–11 at each concentration) were fitted by rectangular hyperbolas with I_{max} of 24.5 pA and an EC_{50} of 0.85 mM (*E*) and with I_{max} of 24.7 pA and an EC_{50} of 0.265 mM (*F*). Curve fittings were made using a nonlinear least-squares method.

et al., 1987; Ohno-Shosaku et al., 1987). Thus, we investigated the requirement of Mg^{2+} ions for AlF complex-induced activation of NS channels in endogenous ATP-depleted cells where 2 mM ATP was exogenously supplied. Figure 2*C* depicts a whole-cell current recorded with the standard AlF solution in which $MgCl_2$ was absent. An inward I_{NS} began to develop 79 ± 10 sec (n = 3) after break-in, and reached a maximum of 17.7 ± 5.2 pA at 324 ± 50 sec. This time course and maximum amplitude of I_{NS} were not significantly different from those seen in the presence of 12 μM Mg^{2+}, under similar conditions (latency, 54 ± 13 sec, n = 5; amplitude, 21.5 ± 7.2 pA, n = 5; time of maximum, 278 ± 40 sec, n = 5). However, when 5 mM EDTA, which has a high chelating potency for Mg^{2+} ions, was added to a nominally Mg^{2+}-free AlF solution, generation of I_{NS} was almost completely abolished (Fig. 2*D* and *E*). This result indicates that Mg^{2+} ions are required for the AlF complex-induced activation of NS channels.

Effects of Non- or Poorly Hydrolyzable Analogues of ATP

Non- or poorly hydrolyzable analogues of ATP are useful to distinguish various ATP-dependent enzyme systems. ATPγS, in which a nonbridge oxygen of the γ-phosphate is replaced with sulfur, can not substitute for ATP in many ATPase reactions, but can serve as a substrate for protein kinases (Eckstein, 1985), but not all (Parente et al., 1992), whereas AMP-PNP is not utilized for either protein kinase or ATPase reactions (Yount, 1975). AMP-CPP, in which α, β bridge oxygen of the phosphates is replaced with a methyl group, can potentially act as a high energy phosphate donor in some enzymatic reactions (Raymoure et al., 1986) and was reported to support CFTR Cl^- channel activity in CFTR-transfected 3T3 fibroblasts (Anderson et al., 1991). To study effects of these ATP analogues, the analogue was added to a nominally Mg^{2+}-free AlF solution since the binding constant of ATPγS for Mg^{2+} ions has not been reported apparently. Figure 6*A* shows a whole-cell current recorded with 2 mM ATPγS-containing AlF solution in ATP-depleted cells. After a transient outward current subsided, the current level remained almost at zero for 3 min, indicating no development of I_{NS}. Similarly, I_{NS} was not induced in the presence of 2 mM AMP-PNP (*B*). On the other hand, the small amplitude of I_{NS} appeared to be produced with substitution of AMP-CPP for ATP (*C*): an inward current began to develop with a latency of 22 sec and then reached a maximum of 3.8 pA at 190 sec.

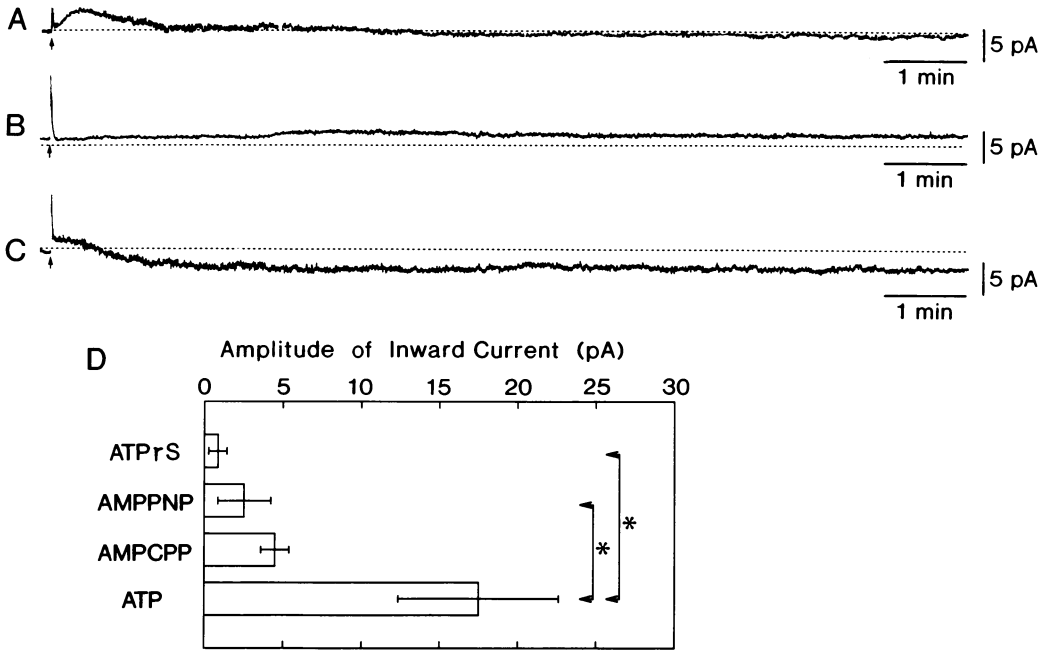

Fig. 6. Effects of replacement of ATP with hydrolsis-resistant analogues on generation of I_{NS} induced by the AlF complex. (*A*), (*B*) and (*C*) whole-cell currents recorded with AlF solutions containing ATPγS, AMPPNP, and AMPCPP, respectively. No $MgCl_2$ was added to AlF solutions and 2 mM ATP was replaced equimolarly with one of the analogues. Endogenous ATP was depleted with cyanide. (*D*) summary of amplitudes of whole-cell currents recorded with AlF solutions containing ATPγS ($n = 4$), AMPPNP ($n = 4$), AMPCPP ($n = 3$), or ATP ($n = 3$). No $MgCl_2$ was added to AlF solutions. Amplitudes of whole-cell currents were measured 3 min after break-in with reference to the current level at 30 sec. *represents statistical significance ($P < 0.05$).

Figure 6*D* summarizes peak amplitudes or amplitudes of current at 3 min measured from an initial current level. Data obtained with ATPγS and AMP-PNP, but not with AMP-CPP, significantly differed from those with ATP under nominally Mg^{2+}-free conditions, whereas data with either of the three ATP analogues were not significantly different from those (3.3 ± 0.9 pA, $n = 4$) obtained with ATP-free AlF solutions. Thus, we conclude that hydrolysis of ATP is required for the AlF complex-induced activation of NS channels.

Nucleotide Specificity

Serine/threonine protein kinases with a broad spectrum of substrates, such as cAMP-dependent kinase, had the high specificity for nucleotide (Edelman, Blumenthal & Krebs, 1987), and a similar specificity was noted for the Na^+, K^+ ATPase (Glynn & Hoffman, 1971). On the other hand, other serine/threonine protein kinases, such as casein kinase II (Baydoun et al., 1981), utilized GTP almost as well as ATP to phosphorylate a target protein. Furthermore, CFTR Cl^- channels were stimulated by a broad array of hydrolyzable nucleotides (Anderson et al., 1991). Thus, we investigated nucleotide specificity for AlF complex-induced I_{NS} in endogenous ATP-depleted cells. The upper panel in Fig. 7 shows whole-cell currents recorded with AlF solutions containing 2 mM GTP (*A*), 2 mM ITP (*B*), and 2 mM UTP (*C*), in which Mg^{2+} ions were kept at 12 μM by adding an appropriate amount of $MgCl_2$. It is evident that GTP, ITP, and UTP could not substitute for ATP in the generation of I_{NS} (Fig. 7*D*).

Effects of H-7

To explore a role for ATP in activation of NS channels, effects of the isoquinolinesulfonamide derivative, H-7, on AlF-induced I_{NS} were examined. Based on our thesis, the inhibition of H-7 could be accounted for by a shift of balance between protein kinase and Mg^{2+}-dependent phosphatase activity toward the latter. Thus, the extent of H-7 inhibition should be diminished by a decrease in Mg^{2+}-dependent phosphatase activity. Figure 8*A* shows I_{NS} in the presence of 100 μM Mg^{2+} in an ATP-depleted cell. Bath application of 100 μM H-7 4 min after break-in led to a 55% suppression of I_{NS} and a similar extent of inhibition was observed with the second application 1 min 50 sec later. However, the third application at 11 min resulted in a 41% inhibition of the current, with a 25% reduction of extent of inhibition. This reduction was further facilitated at 26 min: though the amplitude of the inward current evoked was almost identical with that

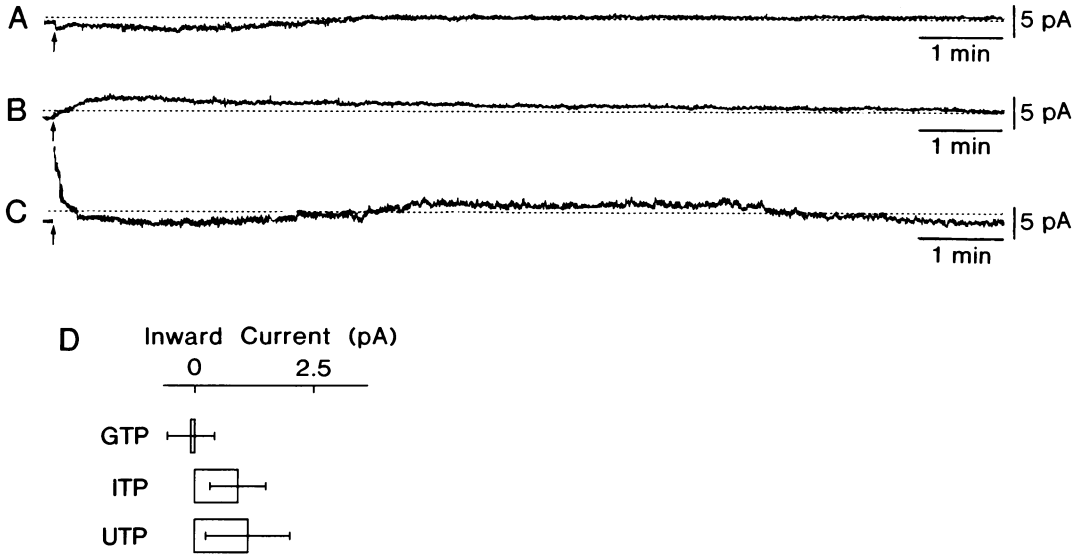

Fig. 7. Nucleotide specificity in generation of I_{NS} by the AlF complex. (*A*), (*B*) and (*C*) whole-cell currents recorded with AlF solutions containing 2 mM GTP (A; $n = 3$), 2 mM ITP (*B; n* = 3), and 2 mM UTP (*C; n* = 3) for ATP, respectively. [Mg^{2+}] in all AlF solutions, 12 μM. Endogenous ATP was depleted with cyanide. (*D*) summary of amplitudes of whole-cell currents recorded with AlF solutions containing one of the nucleotides. Amplitudes of current was measured in the same manner as in Fig. 6.

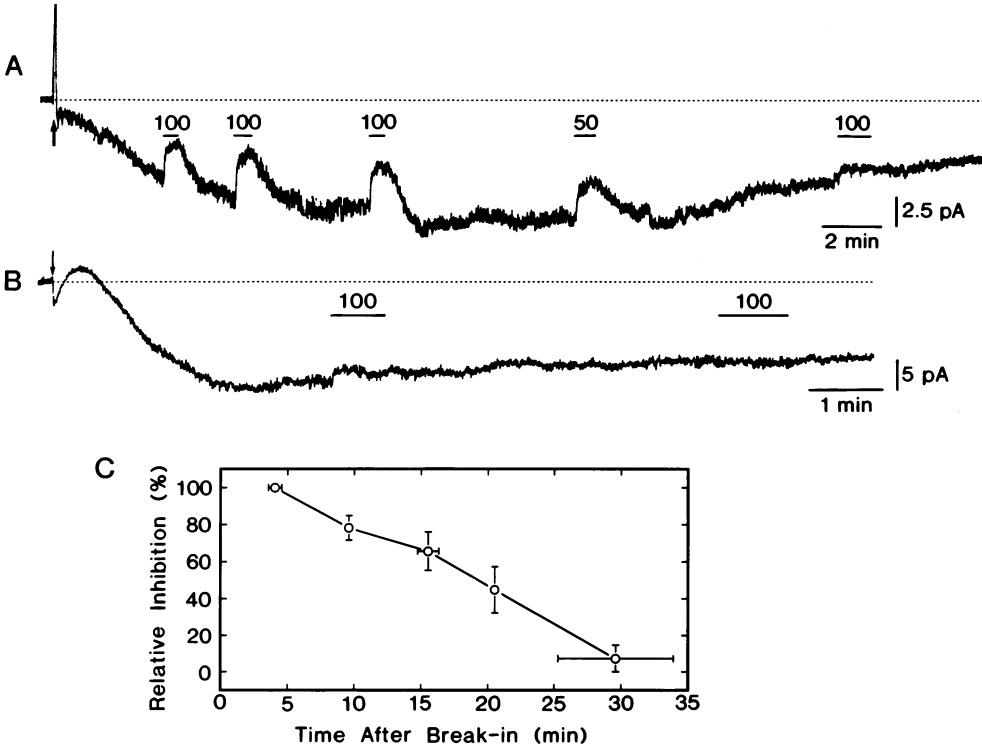

Fig. 8. Time- and Mg^{2+}-dependence of H-7 inhibition of AlF complex-induced I_{NS}. (*A*) and (*B*) whole-cell currents recorded with standard AlF solutions containing 100 μM and 12 μM Mg^{2+}, respectively. H-7 at the concentrations indicated in μM was added to perfusate during the periods (bars). Endogenous ATP was depleted with cyanide. (*C*) relative extents ($n = 3$–5 at each data point) of inhibition of I_{NS} by 100 μM H-7 are plotted against the time after break-in. Extent of inhibition by H-7 is expressed as a fraction of the maximum inhibition. I_{NS} was induced by dialysis with standard AlF solutions containing either 100 μM or 12 μM Mg^{2+} in ATP-depleted cells.

at 4 min, the current was suppressed only by 13% on exposure to H-7, a value which was about 24% of that measured at 4 min. Figure 8*C* summarizes the time dependence of the inhibitory potency of H-7, which was expressed as a fraction of that measured about 4 min after break-in. H-7 lost inhibitory potency almost completely during a 30-min recording. This inhibitory potency of H-7 was markedly reduced by decreasing [Mg^{2+}] in the pipette solution to 12 μM (Fig. 8*B*). Addition of 100 μM H-7 to the perfusate 4 min after break-in produced initially an 11% decrease in I_{NS} and the value diminished to 4% at the end of application (40 sec). The second application of H-7 at 9 min failed to induce inhibition. In nine cells tested in the presence of 12 μM Mg^{2+}, bath appliance of 100 μM H-7 at 3 to 6 min after intracellular access suppressed I_{NS} by 14.3 ± 3.5%, and this value was significantly smaller than the 35.0 ± 4.8% ($n = 6$) at the corresponding time in the presence of 100 μM Mg^{2+}.

Source of ATP: Glycolysis or Oxidative Phosphorylation

The experiments with CN pretreatment suggest that oxidative phosphorylation provided ATP to NS channels under aerobic conditions. If this mechanism is involved in the generation of I_{NS} by dialysis with ATP-free AlF solution, then mitochondria should be present just under the plasma membrane. Electron microscopy of chromaffin cells, however, revealed that mitochondria were clustered near the nucleus, but not under the plasma membrane (Fig. 9). To quantitatively investigate localization of mitochondria in a cell, numbers of mitochondria present within a distance of 1 μm from the nucleus and from the plasma membrane were measured. In 10 cells with more than 15 mitochondria, 52.1 ± 3.4% of them were present near the nucleus and 21.8 ± 3.4% near the plasma membrane. Half of the latter were also within 1 μm from the nucleus as it was eccentrically situated.

Glycolysis is known to be enhanced by an increase in ADP concentrations (Conn & Stumpf, 1976). Thus, glycolysis may be so facilitated under suppressed conditions of mitochondria that glycolytic ATP is probably able to support part of NS channel activity. To explore this possibility, 10 mM glucose was added to CN solution and cells were pretreated for 10 min before intracellular access. Figure 10*A* shows I_{NS} with the largest amplitude induced under such conditions. The dialysis with ATP-free AlF solution began to induce I_{NS} with a latency of 12 sec. This current reached a maximum of 24 pA at 240 sec and then diminished with a $T_{1/2}$ of 204 sec. Among 5 cells tested, amplitudes of I_{NS} were variable, ranging from 1.8 pA to 24 pA: I_{NS} with $I_{MAX} > 8$ pA developed in three cells, but in the remaining two cells I_{MAX} was less than 5 pA. The overall average of I_{MAX} was 10.2 ± 4.0 pA ($n = 5$). On the other hand, the addition of 10 mM sucrose to CN solution did not aid in production of the current (Fig. 10*B*): the amplitude of inward shift of current was less than 3 pA in all three cells exposed to sucrose (1.9 ± 0.8 pA). Therefore, although the values in the presence of glucose did not significantly differ from those in sucrose ($P = 0.17$: Figure 10*E*), ATP derived from glycolysis is concluded to support part of NS channel activity induced by the AlF complex under anaerobic conditions.

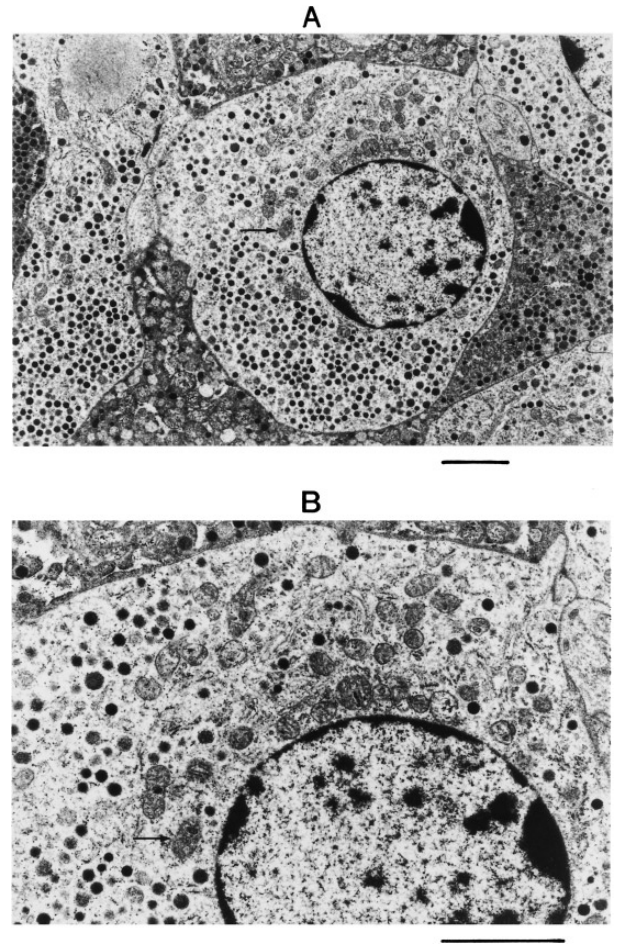

Fig. 9. Electron micrographs of adrenal chromaffin cells. Calibrations in (*A*) and (*B*) 2 μm. (*B*) is a higher magnification of *A*. Arrows show mitochondria and are corresponding in *A* and *B*.

Figure 10*C* depicts AlF complex-induced I_{NS} under conditions where only mitochondrial substrates were available. The cell was first exposed to CN solution for 10 min to deplete cellular ATP and then was incubated in DG solution supplemented with 5 mM pyruvate for 10 min. Infusion of the AlF complex into such cells consistently produced I_{NS} with a I_{MAX} of 7.4 ± 0.7 pA ($n = 3$). This generation of I_{NS} was completely suppressed by addition of 5 mM cyanide to the pyruvate-containing DG solution (Fig. 10*D*). The current level 3 min after break-in shifted in the inward direction only by 1.8 ± 0.7 pA (*n*

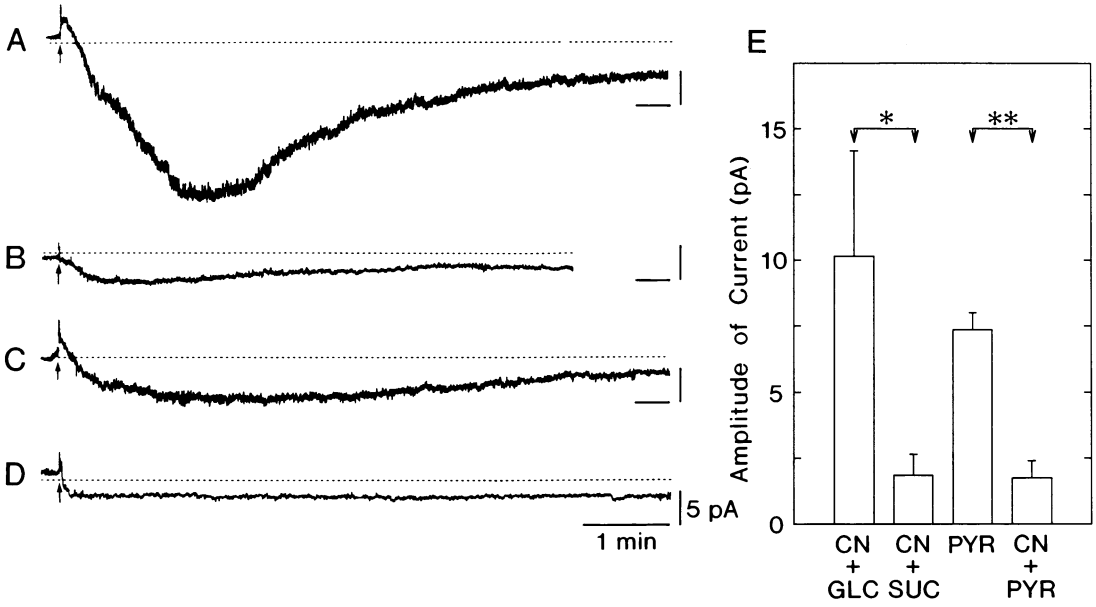

Fig. 10. Glycolytic and mitochondrial ATP support generation of I_{NS} by the AlF complex. (*A*) and (*B*), whole-cell current records from cells pretreated with 10 mM glucose- and 10 mM sucrose-containing CN solutions for 10–12 min, respectively. 2 mM ATP in standard AlF solution was replaced with 4 mM NaCl. (*C*) and (*D*) whole-cell currents recorded with ATP-free AlF solution. Cells were pretreated in CN solution for 10 min, then incubated for 10 min in the pyruvate solution where glucose in standard solution was replaced with 5 mM pyruvate (*C*) or 5 mM cyanide-containing pyruvate solution (*D*). (*E*) summary of maximum amplitudes of AlF complex-induced I_{NS} or current levels 3 min after break-in with reference to the initial level under the indicated conditions. * and ** represent $P = 0.17$ and $P = 0.004$, respectively.

$= 3$) with reference to the initial level. The amplitudies of I_{NS} in the presence of pyruvate with and without cyanide differed significantly ($P = 0.004$: Fig. 10*E*).

Discussion

Source of ATP

In cardiac myocytes, ATP-sensitive K^+ channels utilized ATP generated by glycolysis preferentially over that by oxidative phosphorylation in mitochondria (Weiss & Lamp, 1989). Similarly, Na^+, K^+-ATPase activity in human red cells (Mercer & Dunham, 1981) and cultured cells (Balaban & Bader, 1984) is closely associated with glycolysis. This functional coupling between ion-transporting activity and glycolysis is thought to reflect a close association of glycolysis-related enzymes with the plasma membrane (Jones, 1986). In the present experiment, however, the addition of cyanide to glucose-free solution almost completely abolished I_{NS} induced by ATP-free AlF solution, and AlF complex-induced I_{NS} was not significantly diminished by removal of glucose or by substitution of DG for glucose, but was suppressed by the addition of iodoacetate to DG solution. These results indicate that oxidative phosphorylation in mitochondria mainly supplies ATP to support generation of I_{NS} by AlF complex under aerobic conditions and that chromaffin cells store a sufficient amount of carbohydrate, which is fueled to mitochondria. The finding that about half of NS channel activity was induced by the AlF complex in the presence of pyruvate, a mitochondrial substrate, may not contradict our notion. Probably, exogenous pyruvate does not reach mitochondria as easily as endogenous one. Our thesis is supported by the finding that with exposure to cyanide, generation of I_{NS} by the AlF complex entirely depended on the concentration of ATP in the pipette solution. This result indicates that concentrations of ATP near the plasma membrane decreased to a value less than 100 μM, suggesting that the present treatment with cyanide diminished contents of ATP near the plasma membrane by about 95% (assuming that cellular ATP concentrations are 2–4 mM). In contrast, when ATP contents in adrenal preparations were measured using HPLC, exposure to cyanide decreased concentrations of ATP by about 6%. A similar discrepancy between ATP content and cellular function was noted in guinea-pig taenia caeci: under anaerobic conditions, tension was decreased to 10% of control without a significant diminution of ATP content (Ishida & Paul, 1989). One possible explanation for such a difference is that diffusion of ATP from mitochondria to the plasma membrane is hindered under metabolically suppressed conditions. This hindrance might be due to changes in intracellular environment, such as a decrease

in pH or an increase in Ca^{2+} concentration (Wang et al., 1994). Alternatively, a large amount of ATP might be stored in non-mitochondrial sites, such as secretory vesicles. (Coupland, 1965; Fig. 9).

Under anaerobic conditions, glycolysis provided part of the amount of ATP for activation of NS channels since the maximum amplitude of I_{NS} induced by the AlF complex in the presence of 10 mM glucose and cyanide was about half of that under control conditions. This generation may be due to enhancement of glycolysis under anaerobic conditions.

In the cells in which the machinery for ATP production was little disturbed, the extent of activation of NS channels by the AlF complex was well maintained without an exogenous supply of ATP. This result was unexpected since the concentration of ATP in the vicinity of the channel should have equilibrated with that in the pipette solution within at least a few minutes, based on the diffusion rate calculated with the empirical equation of Pusch and Neher (1988). In fact, the latencies for GTPγS- and AlF complex-induced I_{NS}s were about 60 sec and 30 sec, respectively, and the values were in good agreement with diffusion times calculated for agents used in the experiments. Thus, ATP, but not GTPγS or AlF complex, is thought to be not readily diffusable near the regulatory site of the channel in intact cells. On the other hand, I_{NS} induced by intracellular dialysis with ATP-containing AlF solutions occurred with a latency of about 60 sec in the ATP-depleted cells. This longer latency under metabolically suppressed conditions is consistent with the diffusion time estimated for ATP. This difference between intact and metabolically suppressed cells suggests that oxidative phosphorylation in mitochondria may maintain a local concentration of ATP near NS channels at a level sufficient to support activation of the channel. This possibility, however, may be unlikely since mitochondria were not clustered just below the plasma membrane. Furthermore, in cases where mitochondria were present near the plasma membrane, half of the apparent juxtaposition is probably due to eccentric presence of the nucleus. There is no evidence that a synapse where NS channels are expected to be present is exclusively formed on the plasma membrane which the nucleus is situated near (Tomlinson & Coupland, 1990); thus, the apparent juxtaposition may not reflect the functional association of mitochondria with the plasma membrane. Finally, we might have happened to examine the plasma membrane which lacked NS channels. This notion is not feasible since mitochondria were not consistently observed in the subsynaptic membrane of rat adrenal chromaffin cells (Coupland, 1965). Thus, one possibility to account for the retention is that ATP produced by oxidative phosphorylation is compartmentalized in a ''membrane pool,'' which is resistant to dialysis (*cf.* Mercer & Dunham, 1981).

Mechanisms for ATP Activation of NS Channel

The results with cyanide pretreatment unequivocally demonstrate that ATP is required for the generation of I_{NS} by the AlF complex. This suggests that an ATP-dependent step is present in the signaling pathway from G protein to the channel. However, another interpretation is that depletion of cellular ATP may disrupt the cytoskeleton underlying NS channels, thereby decreasing the number of activatable channels. In various kinds of cells, depletion of cellular ATP with metabolic inhibitors disrupted actin microfilaments (Hinshaw et al., 1993), and this disruption suppressed Cl^- channels in renal epithelia (Suzuki et al., 1993). In fact, actin and actin-binding protein were shown to be linked with several proteins involved in ion transport, such as the α-subunit of the Na^+, K^+-ATPase (Nelson & Veshnock, 1987) and Na^+ channels from the rat brain (Srinivasan et al., 1988). The other possibility is that dephosphorylated channels may lose the potential to respond to a G-protein-mediated signal, similar to pituitary Ca^{2+} channels in a dephosphorylated form that failed to respond to a voltage stimulus (Armstrong & Eckert, 1987). Both possibilities would need to be considered but may not be the case. In our previous study, deactivation of muscarine-induced I_{NS} after washout of the agonist was retarded in the presence of orthovanadate, a nonspecific inhibitor of protein phosphatase or by a decrease in intracellular [Mg^{2+}] (Inoue & Imanaga, 1993). These results suggest that dephosphorylation is responsible for the termination of I_{NS}.

Inclusion in an Mg^{2+}-free AlF solution of EDTA, which has a high chelating potency for Mg^{2+} ions, almost abolished generation of the I_{NS} by the AlF complex. This indicates that trace amounts of Mg^{2+} ions which contaminated the pipette solution and/or were loosely associated with cellular components (Gupta & Gupta, 1984) supported activation of G protein by the AlF complex and probably, a subsequent process leading to openings of NS channels, under nominally Mg^{2+}-free conditions. This interpretation is consistent with the fact that MgATP is a substrate in most ATP-using enzyme reactions, such as phosphorylation or ATPase reactions (Eckstein, 1985). In addition, non- or poorly hydrolyzable analogues of ATP could not substitute for ATP in the activation of NS channels. Therefore, the actual form of ATP involved in activation of the channel may be MgATP, not metal-free ATP, and opening of the channel requires hydrolysis of ATP. The EC_{50} (260 μM) for MgATP probably reflects the interaction between the channel complex and ATP. This EC_{50} approximates K_ms for ATP of phosphorylase kinase (240–380 μM) and myosin light chain kinases (50–400 μM) (Edelman et al., 1987), but is much larger than K_ms of nucleotide-dependent kinases and protein kinase C (3–7 μM), which have a broad spectrum of protein substrates (Edelman et

al., 1987) and have been shown to modulate activity of various ion channels (Levitan, 1994). Furthermore, a similar value of EC_{50} for ATP was reported with respect to CFTR Cl^- channels (230 μM; Anderson et al., 1991). In the latter, energy produced by ATP hydrolysis was proposed to be utilized for a conformational change of channel protein and subsequent openings. Similar values of EC_{50} in activation of CFTR Cl^- channels and NS channels raise the possibility that energy released by ATP hydrolysis is utilized for openings of the latter: i.e., a regulatory site of the NS channel has an ATPase-like activity. However, other hydrolyzable nucleotides could not substitute for ATP in activating NS channels. This high nucleotide specificity for NS channels contrasts with the broad nucleotide specificity observed for CFTR Cl^- channels, where GTP, ITP and UTP can serve as an activator about half as effectively as ATP (Anderson et al., 1991). Furthermore, AMP-CPP substituted poorly for ATP in activating NS channels, whereas it supported three quarters of the activation of CFTR Cl^- channels by ATP (Anderson et al., 1991).

The most pertinent finding indicating the role of ATP as a phosphoryl donor is the reversible inhibition of I_{NS} by H-7. The extent of H-7 inhibition depended on $[Mg^{2+}]$ in the pipette solution and declined gradually during whole-cell recordings. In the case shown in Fig. 8*A*, the extent of inhibition by H-7 decreased from 55% to 13% during the 26 min recording. This decrease could not be ascribed to progressive development of non-I_{NS}, H-7-insensitive current. Infusion of other nucleotide-containing AlF solutions into ATP-depleted cells produced little inward current (1.1 ± 0.5 pA, $n = 13$) during 15 to 30 min recordings. Thus, if an inward current drift as large as 2 pA is assumed, the extent of inhibition at 26 min in Fig. 8*A* would be 21% rather than 13%. This inhibition is still substantially less than the 55% inhibition observed at 4 min. Therefore, the extent of H-7 inhibition is in major part due to loss of the inhibitory potency of H-7 against I_{NS}, and a target site of H-7 is not the NS channel itself. This time-dependent decline of the inhibitory potency of H-7 and its Mg^{2+}-dependence can be readily explained if Mg^{2+}-dependent phosphatase, which is responsible for deactivation of NS channels, washes out during the recording and the degree of the inhibition by H-7 reflects that of the relative dominance of Mg^{2+}-dependent phosphatase over protein kinase (kinase may also wash out during the recording; otherwise, I_{NS} would gradually increase). The observation that ATPγS could not substitute for ATP in the activation may not contradict the phosphorylation hypothesis, since this analogue can not serve as a substrate for protein kinase C (Parente et al., 1992), and even when utilized in phosphorylation reactions, K_ms for ATPγS were large (e.g., cAMP-dependent kinase, 830 μM: Sun, Johnson & Allfrey, 1980). In addition, the slight development of an inward current in the presence of AMP-CPP might be due to activation of NS channels since the γ phosphate of the analogue was released in a putative phosphorylation of estrogen receptors although the cleavage was not so efficient as that of ATP (Raymoure et al., 1986). From the present results, we conclude that phosphorylation of NS channel or its closely associated protein is involved in activation of the channel.

This study was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan and by a grant from Ciba-Geigy Foundation (Japan).

References

Ames, G.F.-L., Lecar, H. 1992. ATP-dependent bacterial transporters and cystic fibrosis: analogy between channels and transporters. *FASEB J.* **6:**2660–2666

Anderson, M.P., Berger, H.A., Rich, D.P., Gregory, R.J., Smith, A.E., Welsh, M.J. 1991. Nucleoside triphosphates are required to open the CFTR chloride channel. *Cell* **67:**775–784

Antonny, B., Chabre, M. 1992. Characterization of the aluminum and beryllium fluoride species which activate transducin: analysis of the binding and dissociation kinetics. *J. Biol. Chem.* **267:**6710–6718

Armstrong, D., Eckert, R. 1987. Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA* **84:**2518–2522

Balaban, R.S., Bader, J.P. 1984. Studies on the relationship between glycolysis and $(Na^+ + K^+)$-ATPase in cultured cells. *Biochim. Biophys. Acta* **804:**419–426

Baydoun, H., Hoppe, J., Freist, W., Wagner, K.G. 1981. The ATP substrate site of a cyclic-nucleotide-independent protein kinase from porcine liver nuclei. *Eur. J. Biochem.* **115:**385–389

Baukrowitz, T., Hwang, T.-C., Nairn, A.C., Gadsby, D.C. 1994. Coupling of CFTR Cl^- channel gating to an ATP hydrolysis cycle. *Neuron* **12:**473–482

Caulfield, M.P., Robbins, J., Sim, J.A., Brown, D.A., Mac Neil, S., Blackburn, G.M. 1991. The naphthalenesulphonamide calmodulin antagonist W7 and its 5-iodo-1-C_8 analogue inhibit potassium and calcium currents in NG108-15 neuroblastoma × glioma cells in a manner possibly unrelated to their antagonism of calmodulin. *Neurosci. Lett.* **125:**57–61

Conn, E.E., Stumpf, P.K. 1976. Outlines of Biochemistry. 4th Edition. John Wiley & Sons, New York

Coupland, R.E. 1965. Electron microscopic observations on the structure of the rat adrenal medulla. II. Normal innervation. *J. Anat.* **99:**255–272

Eckstein, F. 1985. Nucleoside phosphorothioates. *Annu. Rev. Biochem.* **54:**367–402

Edelman, A.M., Blumenthal, D.K., Krebs, E.G. 1987. Protein serine/threonine kinases. *Annu. Rev. Biochem.* **56:**567–613

Flockhart, D.A., Freist, W., Hoppe, J., Lincoln, T.M., Corbin, J.D. 1984. ATP analogue specificity of cAMP-dependent protein kinase, cGMP-dependent protein kinase, and phosphorylase kinase. *Eur. J. Biochem.* **140:**289–295

Furukawa, T., Virág, L., Furukawa, N., Sawanobori, T., Hiraoka, M. 1994. Mechanism for reactivation of the ATP-sensitive K^+ channels by MgATP complexes in guinea-pig ventricular myocytes. *J. Physiol.* **479:**95–107

Glynn, I.M., Hoffman, J.F. 1971. Nucleotide requirements for sodium-sodium exchange catalysed by the sodium pump in human red cells. *J. Physiol.* **218:**239–256

Gupta, R.K., Gupta, P. 1984. NMR studies of intracellular metal ions in intact cells and tissues. *Annu. Rev. Biophys. Bioeng.* **13:**221–246

Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:**5036–5041

Hinshaw, D.B., Burger, J.M., Miller, M.T., Adams, J.A., Beals, T.F., Omann, G.M. 1993. ATP depletion induces an increase in the assembly of a labile pool of polymerized actin in endothelial cells. *Am. J. Physiol.* **264:**C1171–C1179

Inoue, M., Fujishiro, N., Imanaga, I. 1995. Mg^{2+}-dependent phosphatase as an inhibitory mediator of the nonselective cation current induced by aluminum fluoride in guinea-pig chromaffin cells. *Brain Res* **687:**199–204

Inoue, M., Imanaga, I. 1993. Phosphorylation-dependent regulation of nonselective cation channels in guinea pig chromaffin cells. *Am. J. Physiol.* **265:**C343–C348

Inoue, M., Imanaga, I. 1995*a*. Phosphatase is responsible for run down, and probably G protein-mediated inhibition of inwardly rectifying K^+ currents in guinea pig chromaffin cells. *J. Gen Physiol.* **105:**249–266

Inoue, M., Imanaga, I. 1995*b*. Mechanism of activation of nonselective cation channels by putative M_4 muscarinic receptor in guinea-pig chromaffin cells. *Br. J. Pharmacol.* **114:**419–427

Inoue, M., Kuriyama, H. 1991. Muscarinic receptor is coupled with a cation channel through a GTP-binding protein in guinea-pig chromaffin cells. *J. Physiol.* **436:**511–529

Ishida, Y., Paul, R.J. 1989. Evidence for compartmentation of high energy phosphagens in smooth muscle. *In:* Muscle Energetics, R.J. Paul, G. Elzinga, and K. Yamada, editors. pp. 417–428. Alan R. Liss, New York

Jones, D.P. 1986. Intracellular diffusion gradients of O_2 and ATP. *Am. J. Physiol.* **250:**C663–C675

Kihira, M., Matsuzawa, K., Tokuno, H., Tomita, T. 1990. Effects of calmodulin antagonists on calcium-activated potassium channels in pregnant rat myometrium. *Br. J. Pharmacol.* **100:**353–359

Levitan, I.B. 1994. Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.* **56:**193–212

Macara, I.G. 1980. Vanadium-an element in search of a role. *Trends Biochem. Sci.* **5:**92–94

Mercer, R.W., Dunham, P.B. 1981. Membrane-bound ATP fuels the Na/K pump: studies on membrane-bound glycolytic enzymes on inside-out vesicles from human red cell membranes. *J. Gen. Physiol.* **78:**547–568

Nelson, W.J., Veshnock, P.J. 1987. Ankyrin binding to (Na^+ + K^+) ATPase and implications for the organization of membrane domains in polarized cells. *Nature* **328:**533–536

Noma, A. 1983. ATP-regulated K^+ channels in cardiac muscle. *Nature* **305:**147–148

Ohno-Shosaku, T., Zünkler, B.J., Trube, G. 1987. Dual effects of ATP on K^+ currents of mouse pancreatic β-cells. *Pfluegers Arch.* **408:**133–138

Parente, J.E., Walsh, M.P., Kerrick, W.G.L., Hoar, P.E. 1992. Effects of the constitutively active proteolytic fragment of protein kinase C on the contractile properties of demembranated smooth muscle fibers. *J. Muscle Res. Cell Mot.* **13:**90–99

Perrin, D.D. 1979. Stability Constants of Metal-Ion Complexes: Part B Organic Ligands. Pergamon Press, Oxford

Pusch, M., Neher, E. 1988. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pfluegers Arch.* **411:**204–211

Raymoure, W.J., McNaught, R.W., Greene, G.L., Smith, R.G. 1986. Receptor interconversion model of hormone action: II. nucleotide-mediated conversion of estrogen receptors from nonsteroid binding to the lower affinity binding state. *J. Biol. Chem.* **261:**17018–17025

Sakai, H., Okada, Y., Morii, M., Takeguchi, N. 1992. Arachidonic acid and prostaglandin E_2 activate small-conductance Cl^- channels in the basolateral membrane of rabbit parietal cells. *J. Physiol.* **448:**293–306

Shenolikar, S., Nairn, A.C. 1991. Protein phosphatases: recent progress. *In:* Advances in Second Messenger and Phosphoprotein Research, P. Greengard and G.A. Robison, editors. Vol. 23, pp. 1–121. Raven Press, New York

Sillén, L.G., Martell, A.E., Högfeldt, E., Smith, R.M. 1971. Stability Constants of Metal-Ion Complexes. Supplement 1. Special Publication 25. Chemical Soc., London

Spruce, A.E., Standen, N.B., Stanfield, P.R. 1987. Studies of the unitary properties of adenosine-5′-triphosphate-regulated potassium channels of frog skeletal muscle. *J. Physiol.* **382:**213–236

Srinivasan, Y., Elmer, L., Davis, J., Bennett, V., Angelides, K. 1988. Ankyrin and spectrin associate with voltage-dependent sodium channels in brain. *Nature* **333:**177–180

Sun, I.Y.-C., Johnson, E.M., Allfrey, V.G. 1980. Affinity purification of newly phosphorylated protein molecules: thiophosphorylation and recovery of histones H1, H2B, and H3 and the high mobility group protein HMG-1 using adenosine 5′-O-(3-thiotriphosphate) and cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **255:**742–747

Suzuki, M., Miyazaki, K., Ikeda, M., Kawaguchi Y., Sakai, O. 1993. F-actin network may regulate a Cl^- channel in renal proximal tubule cells. *J. Membrane Biol.* **134:**31–39

Tomlinson, A., Coupland, R.E. 1990. The innervation of the adrenal gland. IV. Innervation of the rat adrenal medulla from birth to old age. A descriptive and quantitative morphometric and biochemical study of the innervation of chromaffin cells and adrenal medullary neurons in Wistar rats. *J. Anat.* **169:**209–236

Wang, G.J., Randall, R.D., Thayter, S.A. 1994. Glutamate-induced intracellular acidification of cultured hippocampal neurons demonstrates altered energy metabolism resulting from Ca^{2+} loads. *J. Neurophysiol.* **72:**2563–2569

Weiss, J.N., Lamp, S.T. 1989. Cardiac ATP-sensitive K^+ channels: evidence for preferential regulation by glycolysis. *J. Gen. Physiol.* **94:**911–935

Wise, B.C., Glass, D.B., Chou, C.-H.J., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F., Kuo, J.F. 1982. Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart: II. substrate specificity and inhibition by various agents. *J. Biol. Chem.* **257:**8489–8495

Yount, R.G. 1975. ATP analogs. *Advances in Enzymology* **43:**1–56